Mast cells and endothelial erosion: Implications for human atherothrombotic disease

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Academic Dissertation

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To my family

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ORIGINAL PUBLICATIONS

I Mäyränpää M, Simpanen J, Hess MW, Werkkala K, Kovanen PT. Arterial endothelial denudation by intraluminal use of papaverine-NaCl solution in coronary bypass surgery. *European Journal of Cardio-thoracic Surgery* 2004; Apr;25(4):560-566.

II Lehtonen-Smeds EMP, Mäyränpää M, Lindsberg PJ, Soinne L, Saimanen E, Järvinen AAJ, Salonen O, Carpén O, Lassila R, Sarna S, Kaste M, Kovanen PT. Carotid plaque mast cells associate with atherogenic serum lipids, high grade carotid stenosis and symptomatic carotid artery disease. Results from the Helsinki carotid endarterectomy study. *Cerebrovascular Diseases* 2005;19(5):291-301.

III Mäyränpää MI, Heikkilä HM, Lindstedt KA, Walls AF, Kovanen PT. Desquamation of human coronary artery endothelium by human mast cell proteases: implications for plaque erosion. *Coronary Artery Disease* 2006; Nov;17(7):611-621.

IV Nuotio K, Mäyränpää MI, Saksi J, Ijäs P, Sairanen T, Carpén O, Soinne L, Saimanen E, Salonen O, Lepäntalo M, Kovanen PT, Kaste M, Lindsberg PJ. Endothelial Apoptosis Does Not Determine Symptom Status in Carotid Artery Disease. *Cerebrovascular Diseases* 2007;23(1):27-34.

V Mäyränpää MI, Reséndiz JC, Heikkilä HM, Lindstedt KA, and Kovanen PT. Improved identification of endothelial erosion by simultaneous detection of endothelial cells (CD31/CD34) and platelets (CD42b). *Endothelium: Journal of Endothelial Cell Research* (In press).

These publications will be referred to hereafter as “Study” with the Roman numerals I – V.

Some previously unpublished data is also presented.

ABBREVIATIONS

AEC	3-amino-9-ethylcarbazole
Apo	apolipoprotein
BM	basement membrane
BTEE	*N*-benzoyl-L-tyrosine ethyl ester
Ca	calcium
CaSMC	human coronary artery smooth muscle cell
CD31	platelet-endothelial cell adhesion molecule 1
CD146	melanoma adhesion molecule
CRP	C-reactive protein
DAB	3,3'-diamino-benzidine
DAPI	4',6-diamidino-2-phenylindole
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
FALGPA	furylacryloyl-Leu-Gly-Pro-Ala
HCAEC	human coronary artery endothelial cell
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
HRP	horseradish peroxidase
Ig	immunoglobulin
IFN-γ	interferon gamma
JAM	junctional adhesion molecule
K	potassium
kDa	kiloDalton
LDL	low-density lipoprotein
LDL-C	low-density lipoprotein cholesterol
Mab	monoclonal antibody
MC	mast cell
MMP	matrix metalloproteinase
mRNA	messenger RNA
Na	sodium
NF-κB	nuclear factor κB
NO	nitric oxide
Pab	polyclonal antibody
PCM	pericellular matrix
PCSK9	proprotein convertase subtilisin/kexin type 9
PGI_2	prostaglandin I_2 (prostacyclin)
PAR	protease-activated receptor
rh	recombinant human
RNA	ribonucleic acid
SEM	scanning electron microscopy
SMC	smooth muscle cell
t-PA	tissue-type plasminogen activator
TNF-α	tumor necrosis factor-α
VCAM-1	vascular cell adhesion molecule 1
VE-cadherin	vascular endothelial cadherin

ABSTRACT

More than 40% of all deaths in Finland are caused by atherosclerosis. The complications of atherosclerosis are due to either detachment of the luminal endothelium (erosion) or rupture of the fibrous cap of an atherosclerotic plaque (rupture). As a result, a thrombus is formed at the site of the intimal lesion. Indeed, erosions cause roughly 40% of sudden atherothrombotic deaths and 25% of all atherothrombotic deaths. Erosions are overrepresented in young subjects, diabetics, smokers and women.

This dissertation focuses on endothelial erosion. Endothelial erosions were studied in the context of arterial grafting and vascular inflammation. Special attention was given to the role of intimal mast cells and the methodological viewpoints of reliable identification of endothelial erosions.

Mast cells are inflammatory cells mostly known for their ability to cause allergic symptoms. In addition to occurring in skin and mucosal surfaces, mast cells are abundant in arterial intima and adventitia. In this study, mast cells were found to associate with endothelial erosions in non-lesional and atherosclerotic human coronary arteries. Thus, mast cells may participate in atherogenesis at the initial phases of the disease process already. We also showed that the mast cell proteases tryptase, chymase, and cathepsin G are all capable of cleaving molecules essential for endothelial cell-to-cell and cell-to-extracellular matrix interactions, such as VE-cadherin and fibronectin.

Symptom-causing carotid plaques were found to contain more inflammatory cells, especially mast cells, than non-symptom-causing plaques. Furthermore, the atherogenic serum lipid profile and the degree of carotid stenosis turned out to correlate with the density of carotid plaque mast cells. Apoptotic and proliferating cells were more abundant in non-symptom causing plaques (active renewal of endothelial cells), but erosions were larger in symptom-causing plaques (capacity of endothelial regeneration exceeded).

The process of identifying endothelial erosions with immunostainings has been ambiguous, since both endothelial cells and platelets express largely the same antigens. This may have caused inaccurate interpretations of the presence of endothelial erosion. In the last substudy of this thesis we developed a double immunostaining method for simultaneous identification of endothelial cells and platelets. This method enables more reliable identification of endothelial erosions.

Keywords: atherosclerosis, endothelium, endothelial erosion, mast cell, tryptase, chymase, cathepsin G

TIIVISTELMÄ (Finnish abstract)

Yli 40 % kaikista kuolemista Suomessa on ateroskleroosin aiheuttamia. Ateroskleroosin komplikaatiot syntyvät suonen sisäseinämää verhoavan endoteelin irrotessa (eroosio) tai ateroskleroottisen plakin rasvaytimen päällä olevan sidekudoskaton revetessä (ruptuura). Sekä eroosion, että ruptuuran seurauksena syntyy verihyytymä. Eroosiot aiheuttavat jopa 40 % kaikista aterotromboottisista äkkikuolemista ja 25 % aterotromboottisista kuolemista. Eroosioiden merkitys kuolinsyynä ruptuuroihin verrattuna on korostunut nuorissa ikäryhmissä, naisilla ja tupakoitsijoilla.

Tässä väitöskirjatyössä on selvitetty papaveriinin ja syöttösolujen osuutta endoteelieroosioiden synnyssä, endoteelieroosioiden esiintymistä oireisten ja oireettomien potilaiden kaulavaltimoplakeissa, apoptoosin merkitystä karotisplakkien eroosioiden synnyssä, sekä pyritty kehittämään luotettava immunohistokemiallinen värjäysmenetelmä endoteelieroosioiden osoittamiseksi.

Syöttösolut ovat tulehdussoluja, jotka tunnetaan yleisesti allergiaoireiden aiheuttajina. Syöttösolujen määrä on suuri ihon ja limakalvopintojen lisäksi myös valtimoiden seinämissä, jossa ne sijaitsevat endoteelin välittömässä läheisyydessä. Tutkimuksessa totesimme, että syöttösolut assosioituvat endoteelieroosioiden kanssa oireettomissa ja jopa lähes terveeltä näyttävissä suonissa ja ne voivat siten osallistua ateroskleroosin kehittymiseen jo sen alkuvaiheissa. Lisäksi totesimme, että syöttösolujen vapauttamat proteaasit tryptaasi, kymaasi ja katepsiini G kykenevät kaikki pilkkomaan endoteelisolujen solu-soluväliaine ja solu-solu adheesion kannalta keskeisiä molekyylejä kuten fibronektiiniä ja VE-kadheriinia.

Totesimme myös, että oireisten kaulavaltimoahtaumapotilaiden kaulavaltimoplakeissa on enemmän tulehdussoluja, erityisesti syöttösoluja, kuin oireettomilla potilailla. Lisäksi havaitsimme, että suurentunut syöttösolumäärä korreloi myös potilaiden aterogeenisen seerumin lipidiprofiilin ja ahtauma-asteen kanssa. Totesimme myös, että oireettomilla potilailla on enemmän apoptoottisia ja jakautuvia endoteelisoluja (suuri vaihtuvuus, ja toimivat korjausmekanismit) kuin oireisilla, joilla puolestaan on suuremmat eroosiot (vähän soluja ja korjausmekanismit eivät toimi).

Endoteelieroosioiden tunnistaminen immunovärjäyksin on ollut epävarmaa, koska endoteelisolut ja verihiutaleet ilmentävät suurelta osin samoja antigeenejä. Tämä on aiheuttanut helposti virheellisiä tulkintoja endoteelin erosoitumisesta tai eroosion puutteestä. Viimeisessä osatyössä kehitimme ratkaisuksi tähän ongelmaan immunohistokemiallisen värjäysmenetelmän, jolla pystyy värjäämään samanaikaisesti sekä verihiutaleet että endoteelisolut ja voi siten tunnistaa endoteelieroosiot aiempaa luotettavammin.

Avainsanat: ateroskleroosi, endoteeli, endoteelieroosio, syöttösolu, tryptaasi, kymaasi, katepsiini G

1. LITERATURE REVIEW

1.1. General introduction

Atherosclerosis is a complex, multifactorial, and multifocal disease of large and medium-sized arteries. Atherosclerotic changes begin to develop early in life and are already visible in children[1, 2]. This development starts at the bifurcations and curvatures of arteries, which are the sites most prone to atherosclerosis.

Initially, atherogenic plasma lipoproteins (LDL, IDL, VLDL) enter the arterial intima and accumulate there. The lipoproteins attach to intimal proteoglycans and become modified by oxidative and proteolytic mechanisms, which then leads to recruitment of inflammatory cells[3, 4]. The inflammatory cells start phagocytosing modified lipids, become lipid-filled foam cells, and potentiate the inflammation. Gradually, the chronic intimal inflammation, accumulation of lipids, and proliferation of intimal cells lead to marked structural changes in the intima, i.e. intimal thickening, fatty streak formation, and eventually the formation of an atherosclerotic plaque with an extracellular lipid core, i.e. atheroma. The changes in the arterial wall usually remain asymptomatic until they reach an advanced stage. Thus, by the time atherosclerosis becomes symptomatic, it is usually widespread, and the lesions causing complications have either eroded or ruptured, causing subsequent thrombus formation and ischemic symptoms.

Changes resembling atherosclerosis are also observed in venous and arterial grafts used for by-pass operations (graft atherosclerosis), at sites of balloon angioplasty (restenosis), in transplanted organs (transplant atherosclerosis), as well as in the arterio-venous fistulae used as hemodialysis access point in patients with renal failure.

1.2. Normal arterial structure

The normal arterial wall is composed of three layers: intima, media, and adventitia[1] (Figure 1). These layers consist of several cell types and extracellular structures, which are briefly presented here.

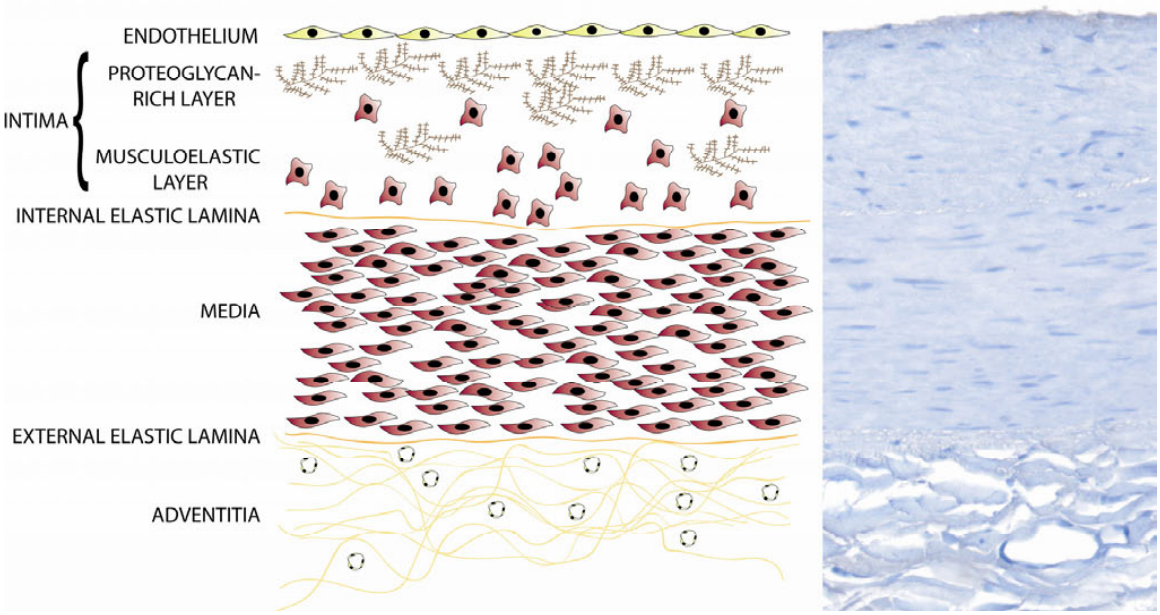

Figure 1. Schematic presentation of the normal arterial wall structure and a corresponding photomicrograph of a hematoxylin-stained healthy human coronary artery.

Intima

The innermost layer of the arterial wall, the intima, is separated from the arterial lumen by the endothelium and from the media by a thin elastic layer called the internal elastic lamina[5]. The endothelium consists of a monolayer of endothelial cells, and it plays a critical role in a variety of functions. It provides a smooth antithrombotic surface and regulates blood flow, blood coagulation, leukocyte adhesion, vascular smooth muscle cell (SMC) growth, and diffusion of liquids and solutes into tissues[6]. The intimal layer can be further divided into a superficial proteoglycan-rich layer and a deeper musculoelastic layer. The main constituents of the extracellular matrix of the superficial layer are type I, III, and IV collagens and the proteoglycans decorin, versican, biglycan, and hyaluronan[7, 8]. The deeper musculoelastic layer contains more SMCs, collagen, and elastic fibers than the superficial layer. The thickness of the intimal layer varies markedly in different parts of the arterial tree. The relative thickness of the intima is small in the large arteries, but greater in the medium-sized arteries and focally at sites of turbulent blood flow, whereas the intima in small arteries is very thin. Generally speaking, areas with thick intima, especially sites adjacent to arterial bifurcations, are prone to atherosclerosis.

Media

The middle layer of the arterial wall, the media, is mainly composed of circularly arranged contractile SMCs and elastic fibers, which are composed of two distinct components, a more abundant amorphous component (elastin) and a microfibrillar component. The media regulates arterial tone by its elastic properties and by active, regulated relaxation and constriction of SMCs. The media can be distinguished from the intima and adventitia by its color and by the transverse arrangement of its fibers and lamellae. The amount of SMCs, elastic fibers, and elastic lamellae is highest in the large arteries, i.e. the aorta and its branches, and decline markedly in smaller arteries. In the small distal coronary artery branches the media consists principally of a few lamellae of plain muscle fibers. The media is the thickest layer of the arterial wall in large and medium-sized arteries, and it thus largely determines the overall thickness of the arterial wall.

Adventitia

The outermost layer of the coronary artery is a layer of connective tissue called the adventitia. It consists mainly of fine bundles of whitish connective tissue arranged into an elastic recoiling mesh. In medium-sized arteries, this mesh is highly elastic and forms a special layer, which can be identified between the other adventitial structures and the media. In addition, the adventitia contains nerves and nutrient capillaries that supply oxygen and nutrients to the media[9, 10]. The nutrient capillaries are called *vasa vasorum*. The relative thickness of the adventitia is small in large arteries, but markedly greater in medium-sized arteries, whereas the smallest arteries have virtually no adventitia. It is of interest that surgical removal of the adventitia leads to a complete loss of luminal endothelium[11].

Vascular extracellular structures

Basal lamina and basement membrane

The basal lamina is a thin layer of specialized extracellular matrix (ECM). Basal laminas are present on the basal side of ECs and epithelial cells and surround a number of cell types. The term ‘basement membrane’ is often confused with basal lamina. However, the term ‘basement

membrane' refers to a combination of the basal lamina and *lamina reticularis* or of two basal laminas, which is visible in light microscopy, whereas the basal lamina and *lamina reticularis* can only be visualized in electron microscopy.

The basal lamina is composed of an electron-dense layer (*lamina densa*) between two electron-lucid layers (*lamina lucida*), and it is commonly ~40-50 nm thick (some basal laminas, such as the glomerular basement membrane may be up to 200nm thick). The basal lamina consists mainly of type IV collagen (the main component of *lamina densa*), laminin (the main component of *lamina lucida*), nidogen (also known as entactin), and perlecan, which are all produced by cells attached to the basal lamina[12, 13].

The two layers of the basal lamina (i.e. *lamina densa* and *lamina lucida*) typically lie on top of *lamina reticularis*, which is synthesized by cells of the surrounding connective tissue and contains type IV collagen.

Anchoring fibers composed of type VII collagen extend from the basal lamina to the underlying *lamina reticularis* and loop around collagen bundles. These anchoring fibers are especially numerous in stratified squamous cells of the skin, which are exposed to high levels of mechanical stress.

Extracellular matrix

The arterial extracellular matrix consists mainly of elastin, collagen, and proteoglycans. In large and medium-sized arteries elastin is the main ECM component. Elastin is initially secreted by vascular SMCs as a soluble ~70 kDa polypeptide, tropoelastin. Following post-translational modification, tropoelastin monomers are aligned on a scaffolding of microfibrils composed of fibrillin and cross-linked by lysyl oxidase and organized into elastin polymers that form a network of insoluble concentric rings of elastic lamellae around the arterial lumen. The elastic lamellae are readily visible in light microscopy, and may also be easily recognized in fluorescence microscopy based on their autofluorescence. It is becoming increasingly evident that elastin and fibrillin do not only provide mechanical support to vessels, but also affect vascular homeostasis by regulating cellular functions and cytokine signaling[14, 15].

Fibronectin, vitronectin, tenascin, and osteonectin are also important components of the vascular extracellular matrix. These molecules are involved in the regulation of cell adhesion, migration, angiogenesis, endothelial permeability, and survival[16-20].

Collagen superfamily proteins play a major role in providing tensile strength to vessel walls. In human arteries, type I and type III seem to be most important[21]. Collagens of different types are involved in cell adhesion, differentiation, migration, and apoptosis, and mutations of collagen genes have been linked with numerous diseases, including arterial diseases[22]. In the arterial wall, collagen is mostly produced by SMCs, but endothelial cells, macrophages, and adventitial fibroblasts also produce collagen. The family of type IV collagen found in basement membranes is heterogeneous in that six different α chains form several kinds of molecules. Thus, the composition of type IV collagen in different basement membranes may vary markedly. Interestingly, the non-collagenous domains of type IV collagen appear to inhibit angiogenesis[23]. Vessel walls also contain type XVIII collagen, from which a 22-kDa C-terminal fragment called endostatin may be proteolytically cleaved. Endostatin is an antiangiogenic zinc protein that binds heparin and heparan sulfate and locates preferentially to vessel walls and basement membranes[24, 25]. It seems to limit arterial wall angiogenesis and LDL retention[26, 27].

Proteoglycans are a class of glycosylated proteins which have covalently linked sulfated glycosaminoglycan side chains (i.e. chondroitin sulfate, dermatan sulfate, heparan sulfate, heparin, keratan sulfate). The protein component of proteoglycans is a core protein,

which directs the biosynthesis of proteoglycan. By now, more than 20 genetically different core proteins have been identified, and the recent implementation of molecular biological methods has made it possible to study each proteoglycan and their sub-domains separately. It is becoming increasingly clear that proteoglycans are highly heterogeneous despite the similarities in their biochemical structure. Thus, the classification of proteoglycans based on size and side chain structure is mainly descriptive[28].

Cells of healthy intima

Endothelial cells

Endothelial cells (ECs) are differentiated simple squamous epithelial cells. Endothelial cells in each vessel type have their specific characteristics, which are largely retained in *in vitro* culture of cells[29-31]. Therefore, it is of utmost importance to use endothelial cells originating from the vessel of interest and to keep in mind that flow conditions, the pericellular matrix, and adjacent cells play a major role in the regulation of endothelial cell physiology, when interpreting the results of *in vitro* experiments[32]. ECs are attached to a thin basement membrane, which separates them from the intima. ECs regulate the influx of inflammatory cells, nutrients, plasma proteins, lipoproteins, and solutes into the intima in response to a variety of stimuli[6]. Endothelial cells also regulate thrombus formation, vascular tone, smooth muscle cell proliferation, and inflammatory cell activation by releasing nitric oxide, PGI_2, t-PA, and other mediators[33]. Taken together, endothelial cells are central to the regulation of arterial wall physiology and thus of seminal importance in the pathogenesis of atherosclerosis.

Smooth muscle cells

Smooth muscle cells (SMCs) are the main cells of the arterial media and regulate arterial tone by constant active constriction. A population of SMCs is commonly also found in the intimal musculoelastic layer of atherosclerotic arteries. In healthy intima, SMCs are particularly numerous in areas that seem susceptible to the development of atherosclerotic lesions[34]. As SMCs can be divided into subgroups according to their origin and characteristics, it is important to use suitable SMC populations in experimental settings[35].

Quiescent contractile-type SMCs are surrounded by basement membrane produced by them. The SMC basement membrane consists of the same components as the EC basement membrane, and it separates the SMCs from the surrounding ECM. Loss of basement membrane integrity leads to a change of the SMC phenotype into the synthetic type[36].

In atherosclerotic lesions, SMCs are also found in the intima, where they proliferate in response to endothelial injury. On the basis of these findings, Russell Ross formulated a “response to injury” theory of atherosclerosis[37]. According to this theory, SMC are thought to be at least partly responsible for the growth of atherosclerotic plaque[38]. The idea of large SMC-rich plaques causing stenosis of the coronary artery is still valid, albeit too simplistic. It is now understood that SMCs are not only harmful, but increase the tensile strength and stability of the plaque and, more importantly, the plaque cap by producing collagen[39] and participating actively in the healing process of asymptomatic plaque ruptures[39]. Indeed, it is now known that SMC-rich plaques may cause anginal symptoms, but are generally less dangerous than SMC-poor plaques, which are more prone to rupture[40]. Recent evidence indicates that the intimal SMCs in undisrupted plaques originate from locally migrating smooth muscle cells and not from circulating SMC progenitor cells[41]. However, circulating monocytes participating in the pathogenesis of intimal hyperplasia may differentiate into SMC-like fibrocytes, which express many SMC markers, including smooth muscle α-actin[42].

1.3. Introduction to atherosclerosis research

Historical perspective

Atherosclerosis has been burdening mankind, at least in cultures with an adequate food supply, since ancient times, as evidenced by the presence of atherosclerotic plaques in the arteries of Egyptian mummies dating back to 1 500 BC[43, 44]. The first known illustrations resembling atherosclerotic plaques can be seen in some of the drawings of the atlas of human anatomy, *De Humani Corporis Fabrica Libri Septem* (On the fabric of the human body in seven books), written by the Flemish anatomist Andreas Vesalius (1514-1564) and published in Basel in 1543 [45]. Johann Friedrich Lobstein (1777-1835) was the first to use the term 'arteriosclerosis' to refer to calcium-containing arterial plaques. However, the first actual scientific theories on the pathogenesis of atherosclerosis were published by Carl von Rokitansky (1804-1878) and Rudolf Virchow (1821-1902) as late as the mid-19th century. They based their findings on the microscopic examination of tissue samples obtained in autopsies[46].

The inflammatory theory was introduced by Rudolf Virchow, who called atherosclerosis by the name 'endarteritis deformans', meaning that there was an inflammatory process within the intima of atherosclerotic arteries. He also postulated that the fibrous thickening evolved as a consequence of reactive fibrosis induced by proliferating connective tissue cells within the intima[45].

The thrombogenic theory (encrustation theory) of atherosclerosis was introduced by Carl von Rokitansky. He proposed that the deposits observed in the inner layer of the arterial wall are primarily composed of fibrin and other blood elements rather than being the result of an inflammatory process[46].

The lipid theory of atherosclerosis was introduced by Nikolai Anitschkov (1885-1964) in 1913, who demonstrated atherogenesis in experimental animals fed a cholesterol-enriched diet[47]. As early as 1914, the deposition of cholesterol crystals in aortic atheromas was described by Ludwig Aschoff (1866-1942)[45].

All the three major theories of atherosclerosis are still valid and supported by a huge bulk of data. Currently, a combination of the above three theories of the pathogenesis of atherosclerosis is considered most accurate[4]. Indeed, at present, dyslipidemia is considered the major causative factor fueling atherogenesis[48], inflammation is thought to act as a central modulator of atherogenesis and a trigger of acute thromboembolic events[4, 49], and thrombus formation plays a crucial role in the pathogenesis of atherosclerosis and especially the generation of acute complications[50]. These three aspects will be discussed in more detail in the following chapters ("Lipids in atherosclerosis" page 16 - 17, "Inflammation in atherosclerosis" page 17 - 27, and "Thrombosis in atherosclerosis" page 28), and they are also presented in figure 2, which illustrates schematically the pathogenesis of atherosclerosis.

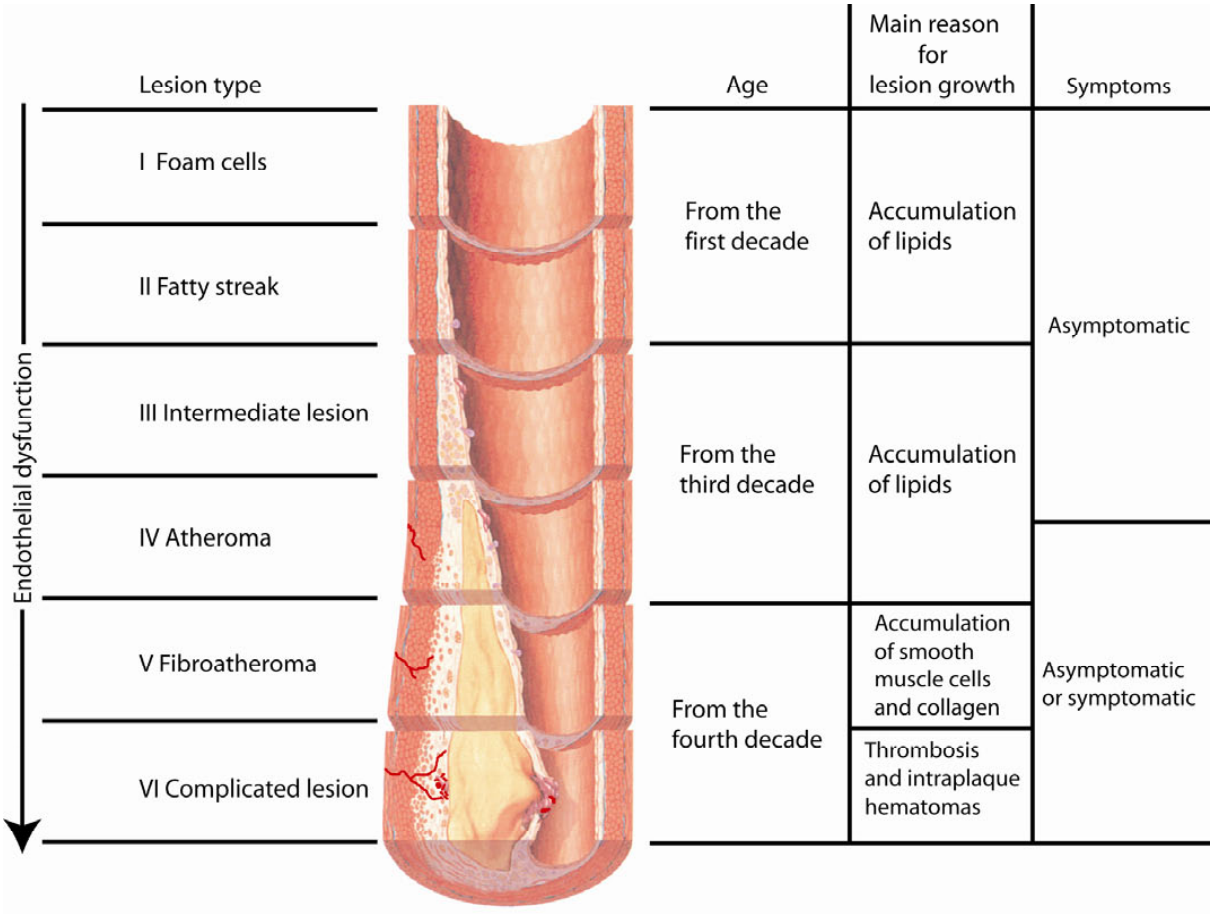

Figure 2. Schematic representation of the progression of atherosclerosis depicting the different types of atherosclerotic lesions, the main pathogenetic factors, and the symptom status related to each lesion type. The importance of endothelial dysfunction from initial to complicated lesions is noteworthy. Accumulation of inflammatory cells starts early in the atherogenesis. Small endothelial erosions may already be present in the early phases of the disease, but larger erosions only appear in the late phases. Neovessels sprouting from the adventitia through the external and internal elastic lamina and media are usually seen only in atheromas and more advanced lesions. Modified from Pepine 1998 [51].

Epidemiology of atherosclerosis

Atherosclerosis is the leading cause of death and disability in Finland and other developed countries[52]. Despite the fact that the number of atherosclerosis-related deaths has been steadily declining since the 1970s in Finland (Figure 3), ~17% of all deaths in the working-aged (aged 14-65) population and >40% of those in the elderly population (over 65) are atherosclerosis-related (Statistics Finland, http://www.stat.fi/). Importantly, the incidence of atherosclerosis is decreasing in the most high-income countries, but rising in the middle- and low-income countries. It has been estimated that the global number of disease-adjusted life years lost due to coronary heart disease will rise from the current ~50 million up to ~80 million by the year 2020. Thus, more efficient means for prevention and treatment are needed.

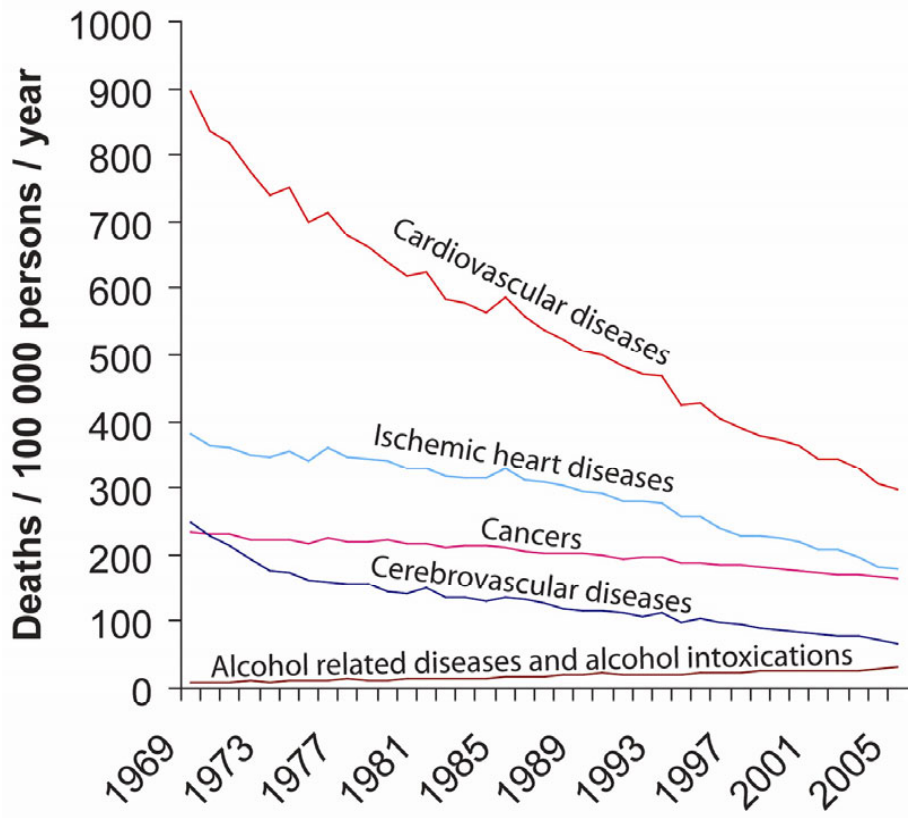

Figure 3. Age-adjusted mortality in Finland for both genders and all age groups, years 1969-2005.
Source: Statistics Finland 31.10.2006.

1.4. Lipids in atherogenesis

Compelling evidence from numerous publications shows that dyslipidemias (i.e. high LDL-C, low HDL-C, and high triglycerides in plasma) are associated with an increased incidence of atherosclerosis[53, 54]. Furthermore, it has been shown that the different lipids and lipoproteins in plasma influence atherogenesis in opposite ways. LDL-C and triglycerides are generally considered harmful, whereas HDL-C is thought to be beneficial. Plasma lipid levels are currently used as one of the variables in combination with other major risk factors when estimating the cardiovascular risk of a patient[55, 56].

A huge body of data also show that modification of plasma lipid levels toward the antiatherogenic direction (i.e. lowering LDL-C, rising HDL-C, and lowering triglycerides) is beneficial and reduces morbidity and mortality regardless of whether these changes are achieved with a diet[57], medication[58, 59], lipoprotein apheresis[60], or surgery[61-63].

The antiatherogenic effects of HDL are numerous, as has been elucidated during the last few decades[64]. The endothelial and antithrombotic effects of HDL have been discussed in a comprehensive review by Mineo *et al.* [65]. Thus, HDL exerts beneficial effects at all phases of atherosclerosis. In line with these observations, an interventional trial where ApoAI was infused intravenously into patients who had severe atherosclerosis showed regression of atherosclerotic plaques within five weeks[66].

Probably the most conclusive evidence of the proatherogenic role of increased LDL-C is the highly increased atherogenesis in patients and animals with inherited familial hypercholesterolemia due to genetic defects of LDL receptors[67]. It has also been shown that even a small reduction in the LDL-C level is highly significant if maintained for the entire duration of the individual's life. This is nicely demonstrated by persons with low LDL-C

levels due to a loss-of-function mutation in the gene encoding PCSK9, which is a serine protease partly regulating the plasma LDL-C levels[68]. The major impact of even slightly reduced LDL-C levels on atherogenesis is understandable in the light of the life-long gradual development of the disease[69]. The gradual slow development of atherosclerotic lesions and the presence of inflammation in the plaques may also explain why even marked reductions in plasma LDL-C have led to only moderate reductions in cardiovascular mortality in some pharmaceutical trials with limited follow-up periods. Indeed, the causal relation between plasma lipids and atherosclerosis is well established, and it can be stated that, if there is no excess lipid (most importantly LDL-C), there is no atherosclerosis. In this sense, atherosclerosis is a "lipid-driven disease"[70]. However, the disease process is greatly modified by inflammation and thrombosis.

1.5. Inflammation in atherosclerosis

As already observed over 150 years ago by Rudolf Virchow, there is an extensive inflammatory reaction in atherosclerotic plaques. The role of inflammation in atherogenesis is supported by the accelerated atherogenesis in patients with autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus[71]. Probably the most convincing evidence highlighting the importance of inflammation in atherosclerosis has been obtained from numerous studies in which the modulation of immunological targets has influenced atherogenesis[72]. Thus, it is clear that inflammation plays a central role in all phases of atherosclerosis[4].

Causes of inflammation in the arterial wall

Numerous factors may induce arterial wall inflammation. Any kind of systemic inflammation will lead to some degree of endothelial activation throughout the vasculature[73, 74]. Similarly, many metabolic factors, such as elevated levels of serum lipids, blood glucose, or blood pressure, are known to induce inflammatory responses in arterial walls. Additionally, local proinflammatory factors in the arterial wall may play a role.

Modified lipoproteins and lipids may also elicit inflammatory responses in the vascular wall[75]. Antibodies against these modified lipids and the T cells reacting with them are found *in vivo*, as they are recognized as "non-self" structures by inflammatory cells. Furthermore, some of these modified lipids resemble bacterial lipids and are recognized by the innate immune system[76, 77].

Epidemiological and experimental studies have provided data linking infections with accelerated atherogenesis and complications of atherosclerosis. The most convincing evidence suggests a proatherogenic role for chronic infections and the total lifetime infectious burden[78]. It also seems clear that acute infections may trigger thromboembolic complications of atherosclerosis[79]. The proatherogenic role of infections is further supported by the observed beneficial effect of vaccines[76, 80]. Thus, despite the result of a recent meta-analysis of antibiotic trials, which did not favor antibiotics as a treatment for atherosclerosis, a potential role of microbial infections in the pathogenesis of atherosclerosis can not be dismissed.

Our bodies are burdened by various toxic substances throughout our lives. Some of these toxins, such as bacterial endotoxins and nicotine from tobacco, are potent activators of inflammatory cells and may hence induce and potentiate vascular wall inflammation[81, 82].

Furthermore, shear stress is known to induce the expression of adhesion molecules on the luminal plasma membrane of endothelial cells, which leads to increased adhesion of inflammatory cells on the endothelium and thus augments the inflammation in the vascular wall[83].

Necrotic and apoptotic cells are also known to initiate inflammatory responses in surrounding cells[77, 84]. Similarly, thrombi and activated platelets are capable of promoting inflammation[85, 86].

Taken together, numerous different and often overlapping mechanisms leading to inflammatory responses play an active role in all phases of atherosclerosis. Importantly, part of the inflammatory processes seem to be beneficial for the host, while part of them are harmful, and some can be considered either beneficial or harmful depending on the context. Thus, it is evident that regulation of inflammation in the arterial wall is of utmost importance in the pathogenesis of atherosclerosis. In studies of arterial wall inflammation, the mechanisms of innate and acquired immunity have been shown to act in concert. The key features of innate and adaptive immunity are briefly discussed below.

Innate immunity in atherogenesis

The mechanisms of innate immunity, which generally work as the fast and blunt first line of defense against pathogens, can also be activated in arterial walls[87]. Innate immunity is evolutionary and ancient, but efficient and fast, and it does not require previous contact with the pathogen. It is constantly primed to act once a non-self structure, such as a pathogen, is encountered. Innate immunity is activated via pattern recognition receptors, including various scavenger and Toll-like receptors. These receptors are developed during the evolution to recognize pathogen-associated molecular patterns. The pathogen-associated molecular patterns are generally highly conserved structures, which are critical for the pathogenicity or existence of pathogens. However, some studies have shown that pattern recognition receptors can also recognize modified self-structures, such as oxidized LDL. It appears that the central components of innate immunity, including complement, phagocytic leukocytes, mast cells, NK cells, and proinflammatory cytokines, may in fact act as the initiators and regulators of the chronic inflammatory response seen in atherosclerotic arteries. Indeed, the termination of innate immune responses is important for the termination of inflammation.

Acute-phase reactants

Acute-phase reactants are important regulators of inflammatory responses. These proteins are mainly produced by the liver, but production has also been shown in peripheral tissues. The expression of these proteins as well as their plasma levels increase rapidly in response to inflammation and tissue injury. Many acute-phase reactants, such as C-reactive protein (CRP)[88] and serum amyloid A[89], have been linked with atherogenesis. Despite extensive research, however, no unambiguous conclusions concerning their role in atherogenesis have been made. Of all acute-phase reactants, only measurement of CRP has been suggested to be included in the cardiovascular risk stratification of intermediate-risk patients[90].

Complement

Complement is mostly known for its capacity to induce lysis of bacteria and cells, and it is generally considered a central mechanism of innate immunity. However, it may also be considered part of the adaptive immune response, as it may be activated by immune complexes via the classical pathway, and it may directly modulate the behavior of cells of the adaptive immune system via cell surface complement receptors[91, 92].

Numerous potential activators of complement, such as immune complexes, modified lipids, cholesterol crystals, CRP, apoptotic cells, and cell debris, are present in atherosclerotic arteries. Additionally, complement activation products, including the terminal membrane-attack complex (C5b-9), have been observed in human atherosclerotic but not healthy

arteries[93]. The complement receptors C3aR and C5aR are also intensely expressed in atherosclerotic areas compared to healthy areas. In addition, numerous complement regulators are present in atherosclerotic lesions and modulate the effects of complement activation in them[94]. Taken together, all the components needed for the activation and regulation of complement are present in atherosclerotic lesions. Interestingly, both pro-and antiatherogenic functions have been attributed to the complement system[95-97].

Riina Oksjoki has summarized the current knowledge of the role of complement in atherosclerosis in a nice and comprehensive way in her thesis entitled "Immune mechanisms in Atherosclerosis: Focus on Complement System" (http://ethesis.helsinki.fi/, ISBN 952-10-3411-4, PDF).

Recruitment of inflammatory cells into atherosclerotic lesions

Recruitment of inflammatory cells into atherosclerotic lesions is an early phenomenon and one of seminal importance in the pathogenesis of atherosclerotic disease. Similar to humans (Figure 4), accumulation of inflammatory cells on the surfaces of atherosclerotic areas has been observed in experimental animals[98].

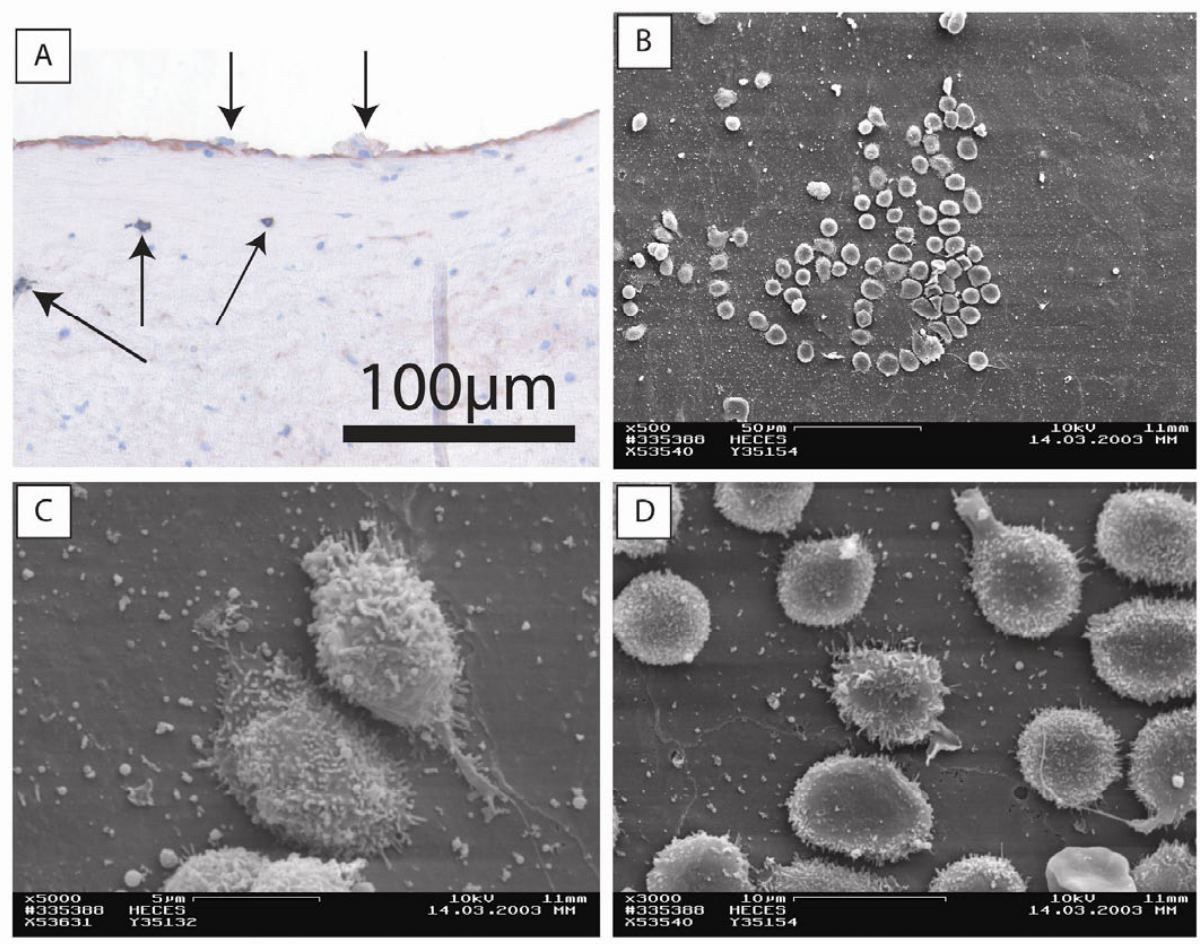

Figure 4. Migration of putative inflammatory cells through the endothelium. Panel A: Tryptase (nickel-DAB, black) and CD31/CD34 (AEC, red) double staining of a human coronary specimen with hematoxylin counterstaining. Tryptase-positive subendothelial mast cells can be seen at the intimal site where putative leukocytes seem to be migrating through the endothelium. **Panel B**: Scanning electron microscopic image of adherent leukocytes on human carotid artery plaque. Leukocytes on carotid and coronary lesions are commonly found as large groups adhering to the endothelium, as seen here. **Panels C and D**: Cells seen in panel B with higher magnifications.

The recruitment of leukocytes into plaques is initiated by the release of chemotactic factors from the cells of the arterial wall[99]. Indeed, numerous such factors have been identified[72, 100]. Once the circulating leukocytes have been primed, they start rolling on the vessel wall. Near the site of chemokine release, the rolling leukocytes adhere more firmly to vascular endothelial cells through a process called "tethering". The tightly adhered leukocytes begin directed luminal migration guided by different guidance cues toward the site of inflammation. Eventually, the leukocytes migrate transendothelially through intercellular spaces and intact cells and across the basement membrane[101]. This transendothelial migration is strictly regulated by bidirectional signaling via the cell surface adhesion molecules on leukocytes and endothelial cells[102]. Furthermore, the leukocytes penetrate through the endothelial basement membrane with a proteolytic machinery, which is likely also to be involved in the penetration of endothelial cell intercellular spaces as well as in the migration through the extracellular matrixes[103].

Macrophages in atherosclerosis

Circulating monocytes, which originate from myeloid bone marrow progenitor cells, enter tissues and mature into macrophages. Macrophages are long-lived cells, which are called by different names in different tissues (e.g. liver macrophages are called Kupffer cells). Macrophages are best known as antigen-presenting phagocytes. In healthy arterial walls macrophages are scarce, but their number increases vastly once an atherosclerotic lesion starts to develop[104]. Indeed, up to 80% of all leukocytes in atherosclerotic lesions are macrophages. Macrophages are known for their ability to phagocytose modified lipoproteins and cellular debris in the arterial wall[105]. As a result of their lipid intake, macrophages turn into lipid-laden cells, which are called foam cells on the basis of their microscopic appearance. Early in atherogenesis macrophage foam cells form the fatty streak lesions in arterial intima. Later in atherogenesis debris derived from necrotic and apoptotic macrophages foam cells are central components of the intimal lipid cores. Macrophages are also capable of producing a vast variety of mediators and are considered the most important type of inflammatory cells in atherogenesis[106].

Neutrophils in atherosclerosis

Neutrophils are bone marrow-derived myeloid granulocytes which normally circulate in blood. Neutrophils have a multilobed nucleus and a cytoplasm filled with granules. There are two main types of granules in neutrophils: 1. azurophil granules containing myeloperoxidase, defensins, cathepsin G, and other mediators; and 2. secondary specific granules containing alkaline phosphatase and lactoferrin. Phagocytosis, killing of microbes, release of cytokines and inflammatory mediators are the main functions of neutrophils. Neutrophils can be stimulated by chemokines, microbial components, and complement.

In blood, the majority of leukocytes are neutrophils, and they also make up the major population of inflammatory cells interacting with endothelium-covering atherosclerotic plaques[98]. Neutrophils enter tissues only upon chemotactic stimuli. In tissues, the life span of neutrophils is short, being only a few days, and the numbers of neutrophils seen in tissues may thus underestimate their importance in slowly developing diseases. In healthy arterial wall solitary neutrophils are rarely seen, but in atherosclerotic areas neutrophils are more commonly observed, and in culprit lesions associated with acute atherothrombotic symptoms neutrophil counts are considerably elevated[107]. Mast cells are known to regulate neutrophil chemotaxis into tissues[108-111]. Interestingly, neutrophil myeloperoxidase is known to be

actively transported via endothelial cells[112] and may cause endothelial erosion[113]. These findings are well in line with the observation that circulating levels of myeloperoxidase seem to predict the risk of future cardiovascular events[114].

Dendritic cells in atherosclerosis

Dendritic cells are potent antigen-presenting inflammatory cells, which have been shown to be present in human atherosclerotic vessel walls. Bone marrow-derived progenitors of dendritic cells and, in some cases, monocytes develop in tissues into mature dendritic cells in response to inflammatory stimulation. Immature dendritic cells are capable of taking up and processing antigens. Activation of these cells by pathogens and inflammatory mediators, leads to their maturation, migration to lymphoid organs, and recruitment of new dendritic cells to the site of inflammation. It has been shown that at least PAR-2 activation by serine proteases (e.g. mast cell tryptase and coagulation factor Xa) is important for the normal maturation process, as PAR-2-deficient mice do not spontaneously develop dendritic cells[115]. Dendritic cells are known to be concentrated in the atherosclerosis-prone areas, which also contain vascular-associated lymphoid tissue[116, 117]. Whether these areas of vascular-associated lymphoid tissue are also enriched in mast cells is not known. On the basis of autopsy studies[117] and animal studies, it is now thought that low-grade chronic inflammation in areas rich in dendritic cells leads to accelerated development of atherosclerosis[118].

Natural killer cells in atherosclerosis

Natural killer (NK) cells are $CD56^+$ large granular mononuclear lymphocytes originating from bone marrow. NK cells are found in circulation and in peripheral tissues, and they are known to act in immune defense against viruses and cancer. The main mechanisms of NK cell action are cytolytic activity via release of cytoplasmic perforin and granzyme-containing granules and production of proinflammatory cytokines. Small numbers of NK cells have been detected in arterial walls throughout the development of atherosclerotic lesions[117, 119].

Mast cells in atherosclerosis

Mast cells were initially suggested to be involved in the pathogenesis of atherosclerosis by Paris Constatinides in 1953 [120]. Soon after this, mast cells were found to be located in the immediate vicinity of coronary endothelial surfaces and were suspected to regulate thrombus formation by releasing heparin[121]. Interestingly, adventitial mast cells also turned out to increase with an increasing degree of atherosclerosis and to be especially numerous in areas of arterial thrombosis[122]. After the initial enthusiasm, mast cells were largely forgotten in atherosclerosis research for decades. Importantly, during this time, significant advances were made in understanding the complex physiology of these cells in other diseases, such as allergies and asthma. Thus, when mast cells re-emerged as a focus of atherosclerosis research in the 1990s, the research community had new tools and a better understanding of mast cell physiology. For instance, the possibility for highly sensitive and specific double immunostainings (Figure 5) made the identification of tissue mast cells easier and more reliable than previously[123, 124].

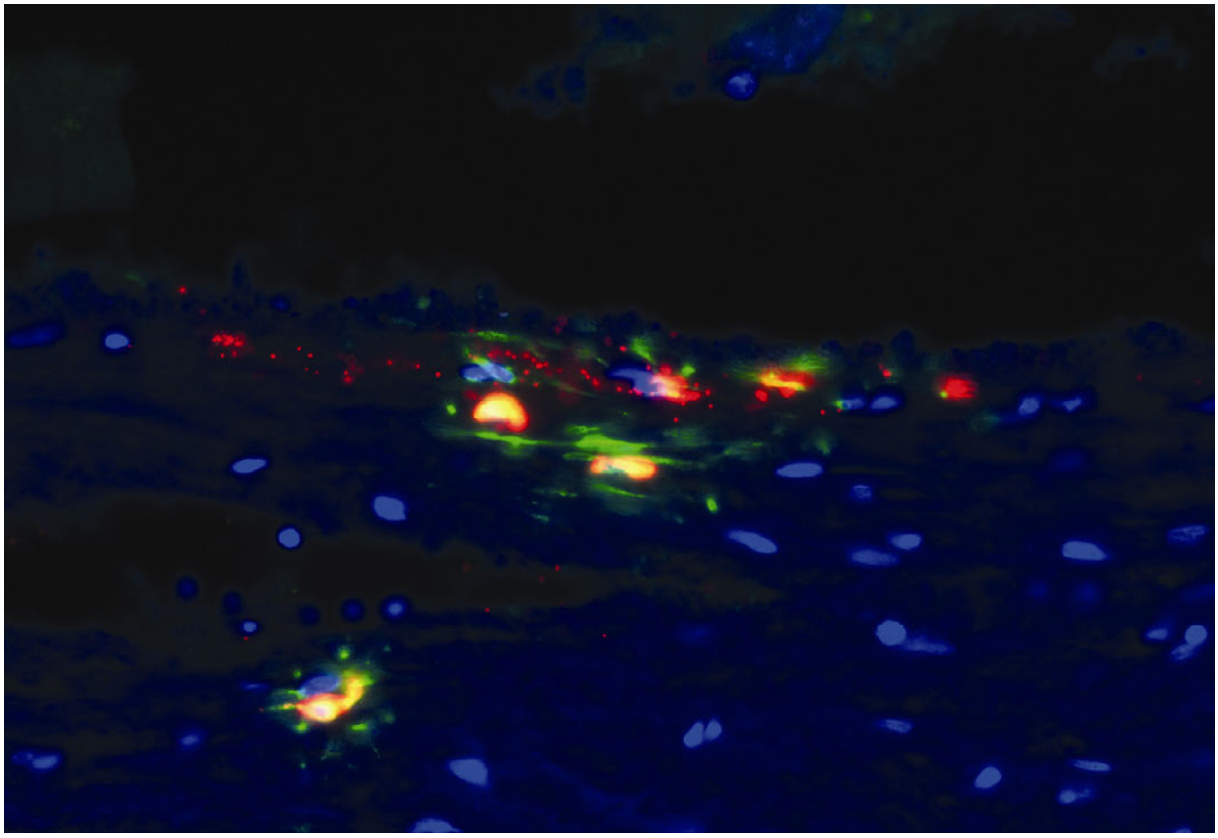

Figure 5. Human carotid mast cells express stem cell factor. A representative immunofluorescence photomicrograph of stem cell factor (green) and tryptase (red) double-positive intimal mast cells in a human carotid artery endarterectomy sample. The patient had suffered a stroke attributable to this carotid plaque 5 days earlier. Note the lack of endothelial cell nuclei, the presence of luminal thrombus, and the numerous extracellular tryptase-positive granules as a sign of recent mast cell degranulation.

Mast cells originate from bone marrow-derived c-kit$^+$ and CD34$^+$ progenitor cells, which are recruited into tissues via poorly described mechanisms. Stem cell factor[125, 126], thrombin[127], chemokines, such as eotaxin[128-130], interleukin-9 [131], and anaphylatoxins C3a and C5a[132] are thought to be important in the recruitment of mast cell progenitors into tissues, and at least stem cell factor has been shown to be important in the development of mature mast cells[133]. Once present in tissues, mast cell progenitors differentiate into T (tryptase-containing) or TC type (tryptase- and chymase-containing) mast cells, depending upon the local tissue environment. The functional roles of these two types of mast cells differ, as does their mediator content[134]. The highest numbers of mast cells are generally observed near the boundaries between the outside world and the internal milieu of the body, such as the skin, the pulmonary mucosa, and the digestive tract, conjunctiva, and nose. Indeed, mast cells appear to act as surveillance antennae of the local microenvironment and direct the immune response by regulating the innate and adaptive immune mechanisms[135]. In addition, mast cells are present in most tissues in the vicinity of blood vessels. Mast cells are probably best known for their capacity to release histamine and to induce flare and wheal reactions seen in the mosquito bites or other hypersensitivity reactions[136]. However, the main functions of mast cells are probably to initiate and regulate the inflammatory response, to defend the host against bacterial and parasitic pathogens, to regulate vascular functions, to participate in wound healing and neovascularization, and to recruit and activate other cells[137-140]. Mast cells are also known to phagocytose[141], participate in hypersensitivity reactions, and play a role in the autoimmune responses involved in a variety of diseases, such as rheumatoid arthritis[142],

Crohn's disease, and ulcerative colitis[143]. Recently, mast cells were shown to be important in the regulation of allograft rejection due to their interaction with regulatory T cells[131]. In animal models mast cells have been shown to protect animals from various endo- and exogenous toxins and carcinogens and to limit tumor growth[144-146].

In human coronary arteries mast cells are located in the adventitia in both healthy and atherosclerotic segments. In infarct-related atherosclerotic coronary segments the adventitial mast cell count is markedly increased[147]. The role of these adventitial mast cells has remained enigmatic. However, it has been speculated that these cells could be stimulated by nervous stimuli and regulate vessel tone and the functions of *vasa vasorum*[9].

Mast cells in healthy human coronary intima are relatively scarce, but their numbers increase as atherosclerosis progresses[148]. The highest intimal mast cell counts have been reported at rupture and/or erosion sites in infarct-related coronary arteries[149]. Plaque mast cells have been shown to be involved in plaque neovascularization[150, 151], to induce smooth muscle cell[152, 153] and endothelial cell apoptosis[154], to induce coronary artery spasm[147, 155], and to promote intimal lipid accumulation and foam cell formation in many ways[156-158]. The role of mast cells in acute coronary syndromes has been recently reviewed in a comprehensive manner by Lindstedt and Kovanen[159].

Importantly, mast cells are known to interact with a variety of cell types, including inflammatory cells and endothelial cells[131, 140, 160]. As the Study III of this thesis concentrates on mast cell-induced endothelial erosion, the mast cell – endothelial cell interaction is discussed here in more detail. Importantly, mast cells are known to regulate multiple functions of endothelial cells. Interestingly, this regulation seems to be bidirectional, as endothelial cells may also regulate the functions of mast cells by releasing nitric oxide and stem cell factor[126, 161]. Mast cell-derived histamine increases the permeability of endothelium by binding to specific histamine receptors (H_1) on the endothelial cell surface. This leads to increased phosphorylation of adherent junction molecules and loosening of VE-cadherin-mediated endothelial-to-endothelial cell adhesion[162-164]. PAR-2 cleavage by mast cell-derived tryptase may also induce loosening of VE-cadherin-mediated cell-to-cell adhesions[165]. Interestingly, it appears that PAR-2 activation may be modulated by mast cell chymase and cathepsin G[166, 167].

Mast cell-derived TNF-α can also activate endothelial cells and induce gene expression and function of the endothelial cell adhesion molecules P-selectin, E-selectin, and VCAM[168]. TNF-α is clinically used to increase endothelial permeability in very low concentrations[169, 170]. This effect has been shown to be mediated via TNF-R1 [171]. Interestingly, the effects of TNF-α may be potentiated by IFN-γ and some pharmacological compounds[172].

Although human endothelial cells express histamine (H_1), TNF-α (TNF-R1 and TNF-R2), and PAR-2 receptors, their presence on the basolateral plasma membranes of endothelial cells has not been demonstrated. Moreover, despite the fact that multiple simultaneous stimuli may lead to cellular responses differing markedly from those seen after a single stimulus[173], the combined effects of all these mast cell-derived mediators have not been studied in detail *in vitro*. However, the available functional data from *in vitro* and *in vivo* studies suggests that receptors for these mediators are present both on the basolateral and the apical plasma membranes of endothelial cells, and that acute degranulation of subendothelial mast cells may lead to endothelial detachment.

Mast cell stabilizers and activators

In tissues mast cells may remain quiescent for long periods, but a stimulus may cause them to release very rapidly the preformed granule mediators in a process called degranulation, and to

start active production of newly generated mediators. Mast cell activation may be inhibited by endogenous mediators and pharmacological compounds. Some of the known mast cell stabilizers and activators are listed in table 1.

Table 1. Summary of mast cell activators and stabilizers.

Effector	Observed mast cell reaction	Reference
Endogenous activators		
Histamine	Tryptase release ↑	174
Immunoglobulins	Degranulation ↑	175
Complement (C5a and C3a)	Degranulation ↑	176, 177
Histamine-releasing factors	Histamine release ↑	178
Lipoproteins	Degranulation ↑	179
T cells (direct contact)	Migration and MMP-9 release↑	180, 181
Cytokines	Degranulation ↑	182
Substance P	Degranulation ↑	183
Serum amyloid A	Cytokine release ↑, Degranulation ↑	184
Endothelin-1	Degranulation ↑	144
Eosinophil cationic protein	Degranulation ↑	185
Eosinophil major basic protein	Degranulation ↑	185
Endogenous stabilizers		
Nitric oxide	Degranulation ↓	161
Prostaglandin E2	Degranulation ↓	186
CD300a ligands	Degranulation ↓	187
CD200R ligands	Degranulation and cytokine release ↓	188
Glucocorticoids	Chemotaxis, number and mediator content ↓. Do not prevent degranulation.	189
Exogenous activators		
Bacteria / Bacterial products	Degranulation ↑	190
Parasites	Degranulation ↑	135, 191
Nicotine	Histamine release ↑	82
Calcium ionophores (e.g. compound 48/80)	Degranulation ↑	192
Alkalization of cell	Histamine release ↑	193
Chinese cobra (Naja atra) snake venom metalloproteinase atrahagin	Histamine release ↑	194
Honey bee and snake venoms	Degranulation ↑	145
Radiographic contrast media	Histamine release ↑	195
Exogenous stabilizers		
Sodium cromolyn	Degranulation ↓	196
Histamine receptor antagonists	Cytokine secretion ↓, tryptase release ↓	174, 197
Leukotriene receptor antagonists	Chemotaxis of progenitors ↓	198
Glucocorticoids	Chemotaxis, number and mediator content ↓. Do not prevent degranulation.	189
ß$_2$-receptor agonists	Degranulation ↓	189
Thiourea derivatives	Leukotriene release ↓	199
Protein phosphatase inhibitors	Degranulation ↓	200
Retinoids	Mast cell proliferation ↓	201
Cannabinoid receptor agonists	Degranulation ↓	202
Prostaglandin E2 receptors agonists	Degranulation ↓	186
Phosphodiesterase (PDE5) inhibitors	Degranulation ↓	203

Mast cell-derived mediators

Several lines of evidence suggest that mast cells have a major role in innate immunity[135, 204]. They are capable of producing an armamentarium of important cytokines and other inflammatory mediators, which have been comprehensively reviewed by Martin K. Church *et al.* in the book Middleton's Allergy Principles and Practice [205]. Mast cells express multiple-pattern recognition receptors, which are thought to be involved in the recognition of broad

classes of pathogens, pathogen-derived molecules, and modified endogenous structures[206, 207]. Interestingly, mice without mast cells seem to be much more susceptible to a variety of infections[208]. Mast cells also play a major role during injury and wound healing, as has been nicely demonstrated by the attenuated neovascularization in mast cell-deficient animals[209].

Preformed mediators (granule contents)

Histamine

Histamine is a biogenic amine commonly known for its ability to induce local itching and swelling at sites of allergic skin reactions. The "flare and wheal" reaction seen after a mosquito bite is a good example. Histamine exerts its functions via four different histamine receptors, simply named as H_1, H_2, H_3, and H_4. The tissue- and cell type-specific differences in the expression of histamine receptors are likely to explain the differential and context-dependent responses to histamine. Currently, receptor subtype-specific pharmacological compounds are widely used for research and for the treatment of a variety of diseases and symptoms. Histamine dilates blood vessels, increases vasopermeability, and activates the endothelium with ensuing translocation of P-selectin to the endothelial cell surface, which leads to induced leukocyte rolling *in vivo*[192]. Taken together, these changes lead to local edema (swelling), warmth, redness, and attraction of other inflammatory cells to the site of histamine release. Histamine also irritates nerve endings, which leads to itching or pain associated with cutaneous mast cell activation. Histamine further induces the expression of tissue factor by smooth muscle and endothelial cells[210], which may promote thrombosis in atherosclerotic arteries. Atherosclerotic coronary arteries contain more histamine than healthy coronary arteries and, paradoxically, react to histamine with strong vasospasm[155].

Proteoglycans

Mast cells release macromolecular acidic heparin proteoglycans. Heparin is antithrombotic and exerts a variety of functions on the neighboring cells. Heparin may also bind lipoproteins and may thus play a role in foam cell formation. Heparin is important for the activity of mast cell serine proteases, as it stabilizes the active tryptase tetramers and protects chymase and cathepsin G from their natural inhibitors[211-213]. Thus, tryptase, chymase, and cathepsin G remain active while bound to heparin in tissues.

Serine proteases

Tryptase

Tryptase is a mast cell-specific serine protease, which is released from mast cells in two different forms, constitutively secreted α-tryptase and ß-tryptase released by degranulation[214]. The ß-tryptase is active when bound to heparin in a tetrameric form, but an active monomeric form has also been described[215]. Interestingly, there is no known natural inhibitor of the tetrameric form of ß-tryptase[216], but the reassembly and activity of monomers can be inhibited by a variety of endogenous inhibitors[215]. Furthermore, there seem to be differences in the tryptases of different tissues[217]. Mast cell tryptase has been recently reviewed in a comprehensive manner by Hallgren and Pejler[218].

Chymase

Chymase is a serine protease secreted exclusively by mast cells. It appears that chymase plays a major role in the proteolytic activation of MMP-2 and MMP-9 [219] as well as in the

physiologic degradation of fibronectin and thrombin in tissues[220]. Chymase may act as an angiotensin-converting enzyme[221].

Cathepsin G

The serine protease cathepsin G is an elastolytic angiotensin II-forming enzyme, which is released by neutrophils and monocytes/macrophages in addition to mast cells[82]. Cathepsin G cleaves effectively many components of the extracellular and pericellular matrix, including fibronectin and vitronectin[82].

Carboxypeptidase A

Mast cell carboxypeptidase A seems to be the least thoroughly studied mast cell protease. Its expression in human mast cells has been reported to be limited to TC type cells[222].

Newly formed mediators

Activated mast cells may mobilize arachidonic acid through cytosolic phospholipase A_2, with ensuing rapid generation of both prostaglandin D_2 and leukotriene C_4, the parent molecule of cysteinyl leukotrienes. These eicosanoids act via their respective receptors and are known to serve diverse functions in broncho- and vasoconstriction, cell trafficking, antigen presentation, immune cell activation, matrix deposition, and fibrosis[223]. Interestingly, atherosclerotic arteries are hyperresponsive to the constricting effects of leukotrienes[224]. In addition to eicosanoids, mast cells are capable of producing a variety of interleukins, cytokines, and growth factors[139, 225].

Acquired immunity in atherogenesis

Acquired immunity is crucial whenever the pathogen is not eliminated by the innate immune systems. This may be the case if the pathogen is not recognized by the innate immune system or is able to escape the attacks of innate immunity. The acquired immune system remembers the pathogens it has previously encountered and is also capable of producing a more potent and more specific response to a given pathogen than the innate immune system. Thus, the acquired immune system provides good protection against reinfection by the pathogens the body has encountered before.

Innate immunity is important for the activation of the acquired immune system, the regulation of its responses, and the clearance of antigens targeted by an adaptive immune response. Acquired immunity is largely dependent on the efficient production of antigen-specific high-affinity antibodies and on the mechanisms of cell-mediated immune responses.

B cells in atherosclerosis

In adults, B cells are bone marrow-derived $CD19^+$ and $CD20^+$ lymphoid cells. B cells are mostly known for their ability to produce antibodies, to mediate humoral immunity, to act as antigen-presenting cells, and to participate in lymphoid tissue development. B cells may be stimulated by foreign and self-antigens, IL-4, IL-10, and IFN-γ. B cells can be divided into subsets of type 1 and 2, memory B cells, and plasma cells.

Millions of progenitor B cells are produced daily. These progenitors mature through several stages, which all involve different genetic rearrangements in the antibody-producing genes[226]. During the maturation process, autoreactive and failing immature cells mainly undergo apoptosis. Once a B cell is mature, it will join the long-lived circulating pool of B cells. As a result of genetic changes, all circulating B cells are unique in their ability to bind antigens, as their B cell receptors are all different. These circulating mature B cells do not

produce soluble antibodies, but do express membrane-bound IgM and IgD. In contrast to T cells, which are able to recognize their cognate antigen in a processed form, i.e. as a peptide in the context of an MHC molecule, B cells recognize their cognate antigen in its native form. When a circulating B cell encounters and recognizes its cognate antigen, it internalizes the antigen. Once in the B cell, the internalized antigen is processed and eventually re-expressed on the cell surface MHC class II molecule to a T helper cell for recognition. The activated T helper cell then provides costimulatory signals to the B cell, which enable it to further differentiate into a memory B cell or a plasma cell. The functional CD19-complex on B cells appears to be crucial for this augmentation of the immune response and the formation of immunological memory[227]. This maturation process may take place directly or in the germinal center. In the germinal center, the variable region of B cell antibody hypermutates, and it may change its Ig class. Thus, the cellular machinery generating antibodies is capable of producing an enormously diverse variety of antibodies, despite the fact that only a few genes are involved.

Memory B cells are a special kind of long-lived B cells, which have encountered an antigen once and are primed to activate and produce rapidly large amounts of their specific high-affinity antibody if the antigen is met again.

Plasma cells are efficient secretors of antibodies which bind to their antigens and make them easier targets for phagocytes. The process whereby an antigen is made an easier target for phagocytes is called opsonization. Antibodies also act in concert with the complement cascade, and complement factors and antibodies may together form immune complexes.

B cells have been observed in atherosclerotic arteries, both in intimal lesions and in the adventitial layer[228]. Plasma cells have also been found in plaques[229]. Interestingly, data derived from splenectomized experimental animals has suggested an antiatherogenic role for B cells[230]. In line with this, immunoglobulin genes seem to be highly expressed in stable atherosclerotic lesions compared to unstable lesions, suggesting active local production of antibodies in the lesions and an antiatherogenic role for antibodies[231].

T cells in atherosclerosis

T cells are small mononuclear lymphoid cells, which originate from the thymus and are distributed into the circulation, lymphoid organs, and peripheral tissues. Direct killing of targeted cells and stimulation of cell-mediated and humoral immunity are the main functions of T cells[232]. T cells can be divided into subsets based on their cell surface antigens, i.e. into $CD4^+$ helper and regulatory T cells, and $CD8^+$ cytotoxic T cells. T cells can be found in the arterial wall at all stages of atherosclerosis, and they are considered to play an important role in the destabilization of atherosclerotic plaques[106]. The roles of $CD4^+$ helper, $CD4^+$ regulatory, and $CD8^+$ cytotoxic T cells are currently under intensive investigation in atherosclerosis.

Eosinophils in atherosclerosis

Eosinophils are bone marrow-derived granulocytes, which are known to be involved in allergic disease and in immune defense against parasites. Eosinophils have not been observed in significant numbers in human arterial walls, except around vascular prosthetic grafts and stents made of artificial materials[233] and in some rare cases of eosinophilic vasculitis[234, 235] or hypereosinophilia. The lack of eosinophils in atherosclerotic arterial walls may be related to the fact that eosinophil recruitment, maturation, and survival seem to require a Th2 type inflammatory response, while Th1 type inflammation predominates in atherosclerosis.

1.6. Thrombosis in atherosclerosis

As discussed in the chapter titled "Historical perspective" on page 14, thrombus formation plays an important role in atherosclerosis. The role of thrombosis seems to be biphasic. The fatal and disabling complications of atherosclerosis, such as heart attack and stroke, are caused by arterial thrombosis precipitating on eroded or ruptured atherosclerotic plaque. The role of repeated thrombus formation and the organization of a mural thrombus into the arterial wall in the progression of atherosclerosis is also known as the Duguid hypothesis[236, 237]. Indeed, data derived from patients with inheritable hemophilia and oral anticoagulant therapy, as well as data from animal experiments support the importance of thrombus formation in the development of atherosclerotic plaques even during the clinically quiescent phases of the disease[238-241]. Thus, if unwanted arterial thrombosis could be prevented, atherosclerosis would be a much more benign disease. Importantly, in regard to the pathogenesis of atherosclerosis, the thrombotic theory should not be considered as a separate entity. This is because there is extensive cross-talk between hyperlipidemia, inflammation, and thrombosis[242, 243]. This view is currently also appreciated in the clinical praxis, in which the global vulnerability of a patient is ideally evaluated by considering all aspects of the disease and the patient's other characteristics[244, 245].

1.7. Topic of this thesis – Iatrogenic and mast cell-induced endothelial erosion

This thesis focuses on endothelial erosion. Endothelial erosion was studied in the context of arterial grafting and vascular inflammation. Special attention was given to the role of intimal mast cells and the methodological viewpoints of reliable identification of endothelial erosions.

Pathogenesis of graft atherosclerosis

Graft atherosclerosis affects the arterial and venous grafts used for by-pass operations. Graft atherosclerosis may be considered a healing process of the arterial wall in response to injury. The marked intimal thickening caused by graft atherosclerosis is a clinical problem that sometimes necessitates re-revascularization[246]. The hallmarks of graft restenosis are migration and proliferation of SMCs and deposition of extracellular matrix[247]. It is well established that the major determinants of graft atherosclerosis are: the graft used, the surgical trauma to the graft, altered hemodynamics, and flow shear[248]. Especially, graft endothelial damage appears to be of utmost importance for intimal SMC proliferation[249-251]. Numerous studies have addressed the issue of graft atherosclerosis, and the current consensus is that the smaller the damage to the graft, the better the short- and long-term patency rate of the graft. It is also clear that, in general, the long-term patency of an arterial graft is superior to that of venous grafts[252], and that immunological mechanisms play an important role in the development of graft atherosclerosis[253].

In animal experiments, circulating endothelial progenitor cells have been shown to attach on vascular prostheses and grafts, and this also seems to apply to humans. However, if the formation of the neointima is hindered, endothelialization may also be inhibited.

Endothelial erosions in atherosclerosis

Endothelial erosions have been estimated to account for one quarter of all fatal coronary thromboses[254] and up to 40% of sudden thrombotic coronary deaths[34]. Most endothelial erosions on atherosclerotic lesions are rapidly covered by a white clot composed of activated

platelets with some fibrin and red blood cells. It is evident that most erosions heal unnoticed, and only a few cause any symptoms or acute complications[255]. The likelihood of an erosion to cause symptoms increases if the mechanisms of endothelial regeneration are not fully functional, or if the injury is sustained[256]. Major factors influencing symptom generation by erosions are the thrombogenity of blood and the prevailing flow conditions, which together largely determine the patient's vulnerability[244]. Importantly, the size of the affected artery is also crucial, as the aortic wall may be covered by numerous large ruptured and ulcerated plaques and practically never occludes, whereas only one such plaque is likely to cause a thrombotic occlusion and symptoms in the coronary artery. The mediators released by a platelet thrombus may aggravate the symptoms by causing coronary vasoconstriction, promoting blood coagulation and inflammation, and releasing growth factors, which lead to increased proliferation of underlying smooth muscle cells (SMCs) and to plaque growth[254]. Furthermore, endothelial erosions may cause small infarctions by dispersing microemboli into the microvessels supplied by the affected artery[257]. The microemboli may be hazardous if not resolved and may trigger cardiac arrhythmias or cause transient ischemic attacks or small infarctions of the affected organ. Increased numbers of endothelial cells (ECs) and EC-derived apoptotic microparticles have been found in the circulation of patients with acute coronary syndromes[258, 259]. Thus, EC damage has emerged as a major contributor to the pathogenesis of atherosclerosis and its complications[254].

Comparison of endothelial erosions and plaque ruptures in atherosclerosis

Acute thromboembolic complications of atherosclerosis precipitate via two major mechanisms, namely plaque rupture and plaque erosion[106]. On the basis of autopsy studies, we know that there are differences between patients dying from endothelial erosion and patients dying from plaque rupture. The characteristics of eroded and ruptured culprit plaques are also different. These differences are summarized in table 2.

Etiology of endothelial erosions

Endothelial cell death and endothelial erosion may occur when endothelial cells are exposed to extreme shear stress[259, 260], toxic substances (endotoxin, cytotoxic drugs) [261], unphysiological pH or temperature[262], ischemia (infarction) [263], proteases[264, 265], or mechanical damage (balloon angioplasty) [266]. Inflammatory cells may also cause endothelial erosion by directly killing endothelial cells, as in the case of T cell-mediated EC lysis[267], by releasing free radicals[268], or by degrading endothelial cell adhesion molecules, which leads to detachment or apoptosis of endothelial cells[269].

Protease-mediated endothelial erosion

Many cells are capable of secreting proteases, which may, under suitable conditions, cleave the ECM and PCM components needed for EC matrix adhesion. The cleavage of these anchoring structures may lead either to detachment of living mature endothelial cells or to abolition of EC outside-in signaling and ensuing apoptosis. Regardless of the actual mechanism, the end point is endothelial erosion. The relative importance of these mechanisms of endothelial erosion in the pathogenesis of atherosclerosis is unclear, but supporting evidence for both has been published[269, 270].

Endothelial cell apoptosis as a mechanism of endothelial erosion

Endothelial apoptosis has been suggested to cause large erosions on the basis of animal studies[271], and it is also a potential mechanism of endothelial erosion *in vivo*[272].

Atherosclerotic arteries have apoptotic endothelial cells present, especially in the downstream shoulders of plaques[259], i.e. in the area of turbulent flow unfavorable for endothelial cells[273].

Table 2. Characteristics of patients and culprit plaques of patients who died of myocardial infarction.

	Erosion	Rupture	Reference
Patient characteristics			
Age, study 1, USA	44±7 (n=22)	53±10 (n=28)	274
Age, study 2, Italy	70±9 (n=74)	68±11 (n=217)	275
Smoking	Highly increased risk	Moderately increased risk	276
Atherogenic serum lipids	Moderately increased risk	Highly increased risk	277, 278
Diabetes	Moderately increased risk	Moderately increased risk	277, 279
Hypertension	Moderately increased risk	Highly increased risk	277, 279
Male / female (~% of all cases)	50 / 50	80 / 20	274, 275
Plaque cap characteristics			
Amount of SMCs	High	Low	280
Amount of collagen	High	Low	274, 281
Amount of proteoglycans	High	Low	274
Amount of calcification	Low	High	274, 282
Amount of lipid	Low	High	276
Amount of mast cells	High	High	149
Amount of macrophages	Moderate	High	274, 280
Amount of lymphocytes	Moderate	High	274, 280, 283
Other changes of arterial wall			
Medial atrophy and outward remodeling	Low prevalence	High prevalence	284
Degree of stenosis	Average ~70%	Average ~80%	274
Plaque neovascularization	N.S.	Increased	285
Adventitial inflammation	N.S.	High	147
Amount of adventitial mast cells	N.S.	High	147
Amount of adventitial macrophages	N.S.	High	147
Amount of adventitial lymphocytes	N.S.	High	147, 283
Amount of vasa vasorum	N.S.	High	283

N.S. = not studied

Shear stress and endothelial senescence

Endothelial senescence is thought to take part in the pathogenesis of atherosclerosis. It has been shown in many studies that telomeres are shorter in the ECs in plaque areas and at the predilection sites of atherosclerosis than in other parts of the arterial tree, and that proatherosclerotic factors induce telomere shortening, indicating constant renewal of endothelial cells[286, 287]. It has also been shown that the flow conditions influence profoundly the function of the endothelium and regulate endothelial repair[273, 288, 289]. Interestingly, proatherosclerotic factors seem to induce shortening of the telomeres of circulating endothelial progenitor cells, too[290]. The senescent areas of endothelium may also be stained with a senescent cell marker, β-gactosidase[291]. Flow shear may promote re-endothelialization[292, 293].

Healing of endothelial erosions

Endothelial cells are capable of restoring their functional integrity rapidly after transient non-denuding injury[294], but not after more severe injury, such as balloon-induced denudation[266].

In healthy arteries, denuded areas of endothelium regenerate rapidly within a few hours – a few days by well coordinated migration and proliferation. Re-growth rates up to 0.4 mm/day have been reported in rodents[295]. Moreover, the maximal duration and extent of re-growth seems to be dependent on the species[295]. Unfortunately, no reliable human data is available. Interestingly, proatherogenic factors seem to attenuate endothelial regeneration[296],

whereas the antiatherogenic lipoprotein HDL stimulates endothelial cell proliferation and migration and may thus promote re-endothelialization[297].

Until the finding of endothelial progenitor cells, the repair of damaged endothelium was thought to be mediated by the adjacent EC only. A growing body of evidence suggests that circulating, bone marrow-derived endothelial progenitor cells (EPC) play an important role in EC regeneration. Albeit several bone marrow-derived progenitor cells populations have been shown to participate actively in the re-endothelialization of spontaneous erosions on atherosclerotic plaques, there are marked differences in the capacity of different progenitor populations to act as healers of damaged endothelium[298]. It seems that the relatively undifferentiated $CD34^-/CD133^+/VEGFR\text{-}2^+$ endothelial progenitor cells are especially potent vasoregenerative cells[299]. In patients with atherosclerotic arteries, the numbers of circulating endothelial cells are markedly decreased[300], and the same applies to healthy persons at high risk of cardiovascular disease[290]. In patients with low numbers of endothelial progenitor cells the mechanisms involved in re-endothelialization seem to be malfunctional[301]. It is also known that reduced numbers of circulating endothelial progenitor cells predict future cardiovascular events[302]. Interestingly, at least statin treatment and physical exercise increase the number of endothelial progenitor cells in circulation[303, 304].

There are marked functional and structural differences between regenerated and native endothelium. Regenerated endothelium exhibits impaired endothelium-dependent relaxation, sings of apoptosis, increased uptake of modified LDL, and giant endothelial cells typical of the senescence of endothelial cells[305]. Numerous animal studies have shown that arterial smooth muscle cells under areas of transient endothelial denudation continue increased proliferation even after the completion of endothelial repair[250]. It is also known that arteries with damaged endothelium are prone to develop atherosclerotic lesions later in life[295]. The degree of endothelial and intimomedial damage seems to largely determine the vascular reaction[266, 306, 307]. Indeed, too extensive vessel wall damage, i.e. disruption of the internal elastic lamina or the lipid core, may compromise the results of balloon angioplasty and coronary stenting by promoting local inflammation and restenosis[308]. Currently, restenosis can be effectively prevented by using stents coated with antiproliferative agents[309]. However, the use of coated stents has led to an increasing risk of late in-stent thrombosis, as the antiproliferative agents efficiently prevent the formation of neointima and re-endothelialization of the stented arterial segment[310].

2. AIMS OF THE STUDY

Our group has been studying the role of mast cells in the pathogenesis of atherosclerosis for many years[153, 159, 311]. Our studies have shown that mast cell infiltrates are often associated with endothelial erosions in the culprit lesions that have caused myocardial infarctions[149]. Furthermore, endothelial erosions are known to promote the proliferation of intimal smooth muscle cells[250], and may thus increase the degree of stenosis caused by atherosclerotic plaque, as well as the risk of graft stenosis after coronary artery bypass grafting. In bypass grafting the main factors affecting the graft endothelium are the surgical technique and the antispastic drugs used[312].

At the time when we started this study, the mechanisms of endothelial erosion in atherosclerosis were partly unclear. Furthermore, no morphological data was available on the effect of antispastic drugs on graft endothelium. These two gaps in knowledge motivated us to study the role of papaverine and mast cells in endothelial erosion. The specific aims of this study were:

1. To study the effects of papaverine on arterial graft endothelium at the morphological level.
2. To study the role of mast cells in the pathogenesis of carotid stenosis.
3. To study the association of mast cells and endothelial erosions in human coronary arteries.
4. To study the presence of endothelial erosions in atherosclerotic plaques and the potential mechanisms of endothelial erosion in atherosclerosis.
5. To evaluate and develop methods for studying endothelial erosion.

3. MATERIALS AND METHODS

The methods used in this research project are summarized in Table 3. The methods are described in detail in the original publications. The methods of previously unpublished data are provided in the figure legends showing the data. The antibodies used are listed in Table 4.

Table 3. Methods used in the sub-studies of this thesis

Method	Used in studies	Reference / Supplier
Sample collection and processing for immunohistochemistry	I, II, III, IV, V	
Sample collection and processing for scanning electron microscopy	I, III, IV	
Immunohistochemistry	I, II, III, IV, V	
Scanning electron microscopy	I, III, IV	
Intraluminal treatment of radial arteries with papaverine	I	
Intraluminal treatment of coronary arteries with proteases	III	
Western blotting	III, V	
Determination of endotoxins	III	BioWhittaker
Determination of serum lipids	II	Boehringer-Ingelheim
Computer-aided planimetry	II	Kontron Elektronik
TUNEL assay (ApopTag kit)	IV	Chemicon, Temecula, CA
Trypan blue cell viability assay	I	
Protein quantization (Lowry)	III, V	313
Culture of HCAEC	I, III	PromoCell, Heidelberg, Germany
Platelet preparation and adhesion	V	314
mRNA microarray	IV	Affymetrics

Table 4. List of primary antibodies and non-immune IgGs used in the sub-studies of this research project.

Antibody	Clone / cat. no	Host	Isotype	Working concentration / dilution	Source / reference
Cathepsin G	A0588	Rabbit	Polyclonal	0.22 µg/mL	Dako, Glostrup, Denmark
CD31	JC/70A	Mouse	IgG_1	0.03-3.3 µg/mL	Dako
CD34	QBEnd/10	Mouse	IgG_1	1 µg/mL	Novocastra, Newcastle upon Tyne, UK
CD42b	MM2/174	Mouse	IgG_1	2 µg/mL	Novocastra
CD45RO	UCHL-1	Mouse	IgG_{2a}	4.20 µg/mL	Dako
CD146	N1238	Mouse	IgG_1	0.01-0.1 µg/mL	Novocastra
Cleaved caspase-3	9661	Rabbit	Polyclonal	1:100	Cell Signalling Technology, Danvers, MA
Fibronectin	N-20	Goat	Polyclonal	0.5 µg/mL	Santa Cruz Biotechnology, Santa Cruz, CA
Fibronectin	A0245	Rabbit	Polyclonal	7 µg/mL	Dako
Macrophage	HAM56	Mouse	IgG_1	0.71 µg/mL	Dako
Tryptase	AA1	Mouse	IgG_1	2.2 µg/mL	Dako
Tryptase	G3	Mouse	IgG_1	0.12 µg/mL	Chemicon, Temecula, CA
Tryptase	Eppu	Rabbit	Polyclonal	0.6 µg/mL	315
VE-cadherin	BV6	Mouse	IgG_{2a}	0.1-1.0 µg/mL	Chemicon
Mouse IgG_1 negative control	MCA928	Mouse	IgG_1	Same as primary antibody	Serotec, Oxford, UK
Rabbit IgG	PRABP01	Rabbit	Polyclonal	Same as primary antibody	Serotec

3.1. Human samples

The human samples were obtained from Helsinki University Central Hospital. For Study I, pieces of radial artery were collected during elective coronary bypass operations. All patients were operated in a standardized manner by the same surgeon. Patients with a history of autoimmune disease, radiation therapy, insulin-dependent diabetes mellitus, or cytostatic treatment were excluded, since all these may cause significant endothelial damage and would have hampered reliable evaluation of the papaverine effects.

The studies II and IV are based on carotid plaques collected for the Helsinki Carotid Endarterectomy Study (HeCES). In HeCES, human carotid plaques were collected from carefully characterized consecutive patients undergoing carotid endarterectomy. The patients were referred for endarterectomy according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria. They were thoroughly investigated, and those with other potential causes of cerebral embolism were excluded. In addition to plaques, anamnestic data, laboratory measurements, and imaging results were available for analysis. The characteristics of these patients are described in detail in Study II.

For studies III and V, human coronary arteries were collected from recipient hearts during cardiac transplantation operations.

The study protocols were approved by an institutional ethics committee, and all patients gave informed consent. The study conforms with the principles of the Helsinki Declaration.

The tissue samples used for histology were snap-frozen in liquid nitrogen or fixed with either Carnoy’s fluid or 10% phosphate-buffered neutral formalin. The tissues used for scanning electron microscopy were fixed with 1% glutaraldehyde in Tyrode’s solution perfused with a pressure of 120 mmHg for 60 minutes, after which these specimens were further immersion-fixed with 2.5% glutaraldehyde.

4. RESULTS AND DISCUSSION

4.1. Papaverine-induced endothelial damage (I)

Use of papaverine as a vasodilator in coronary artery bypass grafting

Papaverine is widely used as a vasodilating agent in vascular surgery. Vasodilation is needed for providing suitable conditions for making a surgical anastomosis of arteries[316]. If the anastomosis fails, it may cause bleeding, and if it is too tight, it may cause thrombosis or accelerated occlusion at the sutured site after the operation. Papaverine is commonly administered into the vessel graft. It may also be administered topically or by injection into the pedicle containing the graft vessel[317]. The results presented in Study I show, at the morphological level, that papaverine causes endothelial cell damage when used intraluminally to dilate arterial grafts. Our results suggest that the damage is caused by the low pH of the papaverine solution and by the needle-like papaverine crystals formed in the pH range of 5.3 – 6.0. To further elucidate the mechanisms of papaverine induced endothelial erosions we studied the effects of papaverine on human mast cells which are often associated with endothelial erosions. The unpublished results of these studies show that papaverine induce dose-dependent release of histamine from cultured human mast cells (Figure 6). Morphological evidence suggests that papaverine induce mast cell degranulation (Figure 7) and thus also release of mast cell proteases into the extracellular space. This papaverine-induced mast cell degranulation is an additional mechanism by which papaverine may cause endothelial erosion, as delineated in Study III.

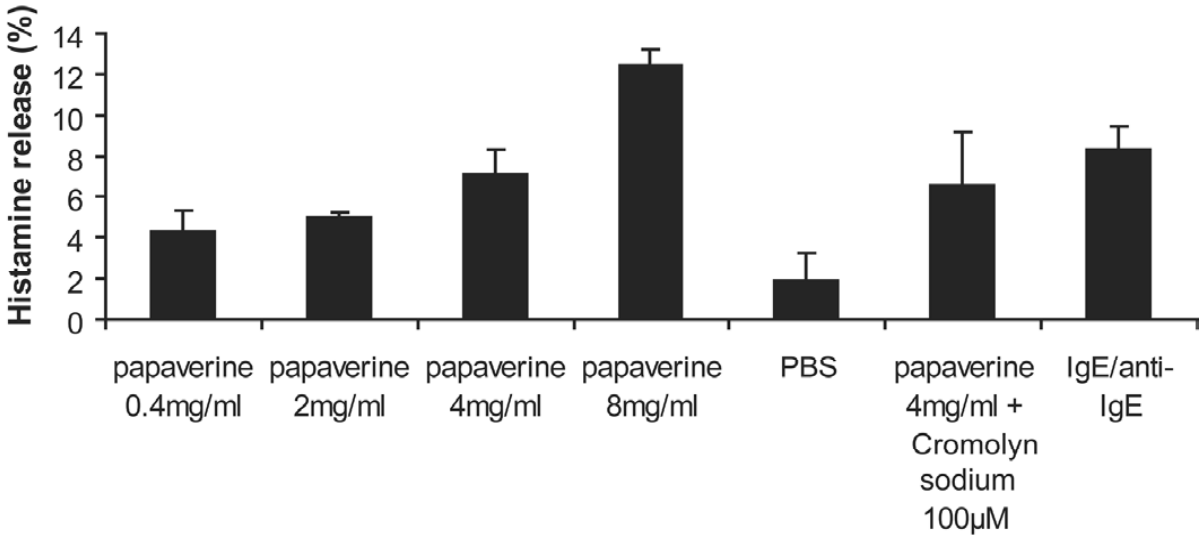

Figure 6. Histamine release (%) from cultured human peripheral blood mast cells in response to increasing concentrations of papaverine. Cells were incubated for 15 minutes with papaverine, and histamine was measured from the media and the cells as previously described, using a fluorometric method[318]. The difference between PBS and 4 mg/ml papaverine was statistically significant (p=0.008, *t*-test). The means of three experiments with standard deviations are shown. Sodium cromoglycate did not markedly inhibit papaverine-induced histamine release. PBS was used as a negative control, and IgE/anti-IgE stimulation as a positive control for histamine release. Mast cells for these experiments were kindly provided by Julia Trosien.

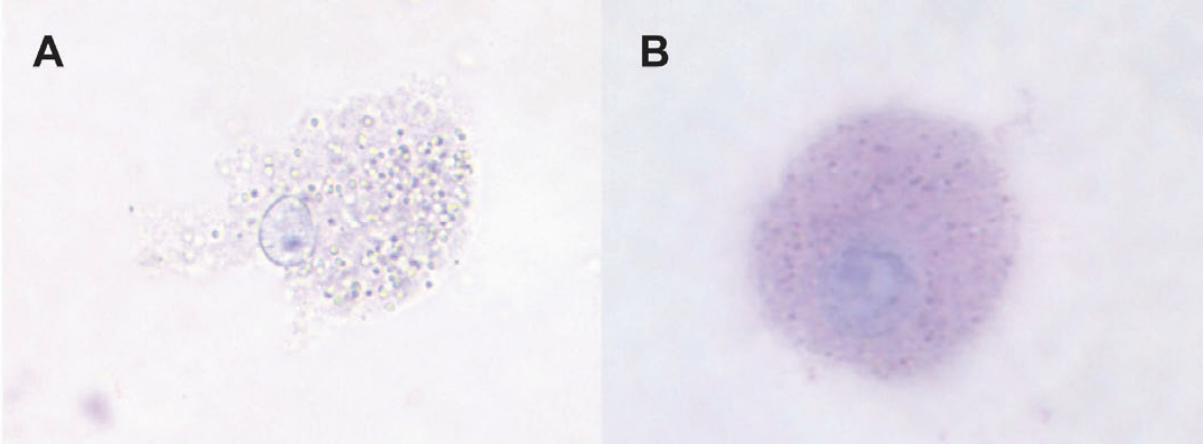

Figure 7. Papaverine-induced mast cell degranulation. Moore & James stained cultured human peripheral blood mast cells. Original magnification for all figures x1000. **A.** Papaverine 4mg/ml induced mast cell degranulation. **B.** Control cells were challenged with PBS instead of papaverine. Degranulation was also observed in samples challenged with lower papaverine concentrations (concentrations ranging from 0.4mg/ml up to 4mg/ml were used). The mast cells for this experiment were kindly provided by Julia Trosien.

Papaverine-induced mast cell histamine release was studied with cultured mature human peripheral blood mast cells. Mast cells were cultured as described in (Lappalainen J. *et al.* 2007, submitted manuscript).

Endothelial erosion can be studied *ex vivo* with two main techniques, namely light microscopy of immunohistochemically stained sections and scanning electron microscopy[319]. In the present study, these two main techniques were applied. In the electron microscopic part of the study, we observed the disruption of endothelial cell plasma membranes, the detachment of endothelial cells, and the opening of endothelial cell-cell junctions. The papaverine-induced endothelial cell damage was also observed *in vitro* in an experiment where cultured human coronary artery endothelial cells (HCAECs) were exposed to papaverine solution and studied afterwards for viability with trypan blue staining. In this experiment papaverine induced the death of a large proportion of HCAECs.

Clinical relevance

The results of the study are in line with the previous reports on the harmful effects of papaverine on vascular graft endothelial cell function[320]. It is evident that such endothelial erosion will eventually heal. Thus, it is difficult to assess the impact of the observed endothelial damage on the patency of the graft. As it is impossible to determine the short- and long-term patency of arterial or venous grafts on the basis of functional or morphological studies, long-term randomized comparative studies are necessary for identifying the most suitable methods for the prevention of graft vasospasm in bypass surgery.

Future research on vasodilators

Future studies should address the following questions:

1. Does graft endothelial damage increase the risk of postoperative thrombotic or ischemic events?
2. Does graft endothelial damage or the use of certain vasodilating compounds increase the risk of graft atherosclerosis in long-term follow-up?
3. Is it possible to reduce the incidence of graft endothelial damage by using vasodilators that are less toxic to endothelial cells?

In order to obtain satisfactory answers to these questions, short- and long-term follow-up

studies are required.

4.2. Mast cell counts are increased in symptom-causing carotid plaques (II)

At the time when this study was initiated, large numbers of mast cells were known to be present in ruptured and eroded human coronary artery culprit lesions[149]. It was also known that endothelial erosions and plaque ulcerations in carotid plaques increase the risk of cerebral thromboembolic complications. Furthermore, elevated intimal mast cell densities in the coronary arteries and the aorta had been previously linked with cerebrovascular thrombi[321], obesity[122], and milky plasma i.e. very high plasma lipid levels[322]. However, no data was available on the presence of mast cells in human carotid arteries or their association with serum lipids.

Mast cell counts are increased in symptom-causing carotid plaques and associate with atherogenic lipid profiles

In this study, we showed that carotid plaque mast cell counts are increased in symptom-causing carotid plaques ($p=0.023$) and in carotid plaques of patients with an atherogenic lipid profile ($p=0.003$). Furthermore, mast cell counts increase in parallel with the degree of carotid stenosis ($p=0.012$), which resembles the findings in coronary arteries (Study III). Indeed, mast cell density correlated positively with serum total cholesterol ($p=0.012$), serum LDL-C ($p=0.013$), and serum triglycerides ($p=0.005$) and negatively with serum HDL-C ($p=0.001$). We also noted that mast cell counts correlated with the density of plaque T cells (r_s:0.40, $p<0.001$). Interestingly, in contrast to carotid and coronary plaques, intimal mast cell counts decreased in atherosclerotic plaques of aorta when compared to healthy aorta[123].

These findings suggest that mast cells participate in the pathogenesis of carotid atherosclerosis. This is apparently the first study to suggest a statistical connection between an atherogenic serum lipid profile and the number of intimal mast cells.

Despite the fact that this study does not provide any mechanistic insight into to the possible role of carotid plaque mast cells, many potential roles can be envisioned on the basis of the previous studies on mast cells. In carotid plaques, mast cells may regulate the actions of other cell types, including T cells[131, 180, 181], neutrophils[323], and endothelial cells[324].

Furthermore, some of the stainings showed infiltrates of activated mast cells under thrombosed endothelial erosions in the carotid plaques obtained soon after the onset of ischemic cerebrovascular symptoms (Figure 5 on page 22), suggesting potential involvement of mast cells in the formation of endothelial erosion. However, as the majority of carotid plaques were obtained a long time after the onset of symptoms (the median time from symptoms to operation was 49.5 days), we were unable to analyze this association of degranulated mast cells with thrombosed carotid plaque erosions statistically.

It has been speculated that the mast cells are recruited to the sites of arterial thrombosis secondary to thrombosis. This is possible, and even likely in cases of chronic wounding and healing as the substances released by thrombi are known to be chemotactic for mast cells[127]. However, the finding that mast cell counts in traumatically lesioned thrombosed arteries are not increased in comparison to healthy areas of the same artery[322] suggests that the factors released by a thrombus are unlikely to be the reason for mast cell accumulation in acute atherothrombotic situations.

4.3. Subendothelial mast cells may cause endothelial denudation (III)

To study the association between subendothelial mast cells and endothelial erosions in healthy and atherosclerotic human coronary arteries we collected and analyzed coronary artery samples obtained from cardiac transplantations. The results of this study suggest that human mast cells may cause endothelial denudation by releasing proteases capable of degrading the components necessary for the EC matrix and EC-EC adhesion, that subendothelial mast cells are an important source of cathepsin G in human coronary arteries, and that mast cells are more numerous than neutrophils in the coronary artery intima.

Subendothelial mast cells associate with luminal microthrombi

We show that subendothelial mast cells associate with the luminal microthrombi covering sites of endothelial erosion even in relatively healthy coronary arteries, and that the proportion of thrombus-associated mast cells increases as atherosclerosis progresses ($p<0.001$). Our immunostainings showed that degranulated mast cells seem to associate with endothelial erosions not only in coronary arteries (Figure 8) but also in carotid arteries, as shown in figure 5 on page 22.

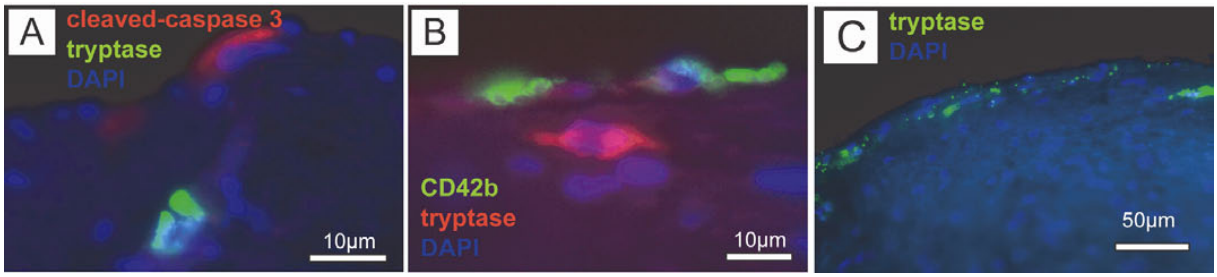

Figure 8. Subendothelial mast cells are associated with endothelial erosions and apoptotic endothelial cells in human coronary arteries. In **A** a cleaved caspase-3-positive apoptotic putative endothelial cell (mouse anti-cleaved-caspase 3 detected with Alexa 596) lies over a subendothelial mast cell (rabbit anti-tryptase detected with FITC). In **B** two platelet microthrombi (mouse anti-CD42b + FITC) lie over a subendothelial mast cell (rabbit anti-tryptase + Alexa 596). In **C** numerous tryptase-positive granules (rabbit anti-tryptase + FITC) are seen spread in the subendothelial intima. Note the lack of endothelial cell nuclei in **C**. **A – C** stained using the antibodies given in parentheses and counterstained with DAPI.

Ex vivo model of mast cell protease-induced endothelial damage

To study the mechanisms potentially involved in mast cell-induced endothelial erosion, we designed an *ex vivo* model in which mast cell proteases were administered intraluminally into fresh human coronary arteries. We were able to show that mast cell proteases may induce endothelial erosion in an *ex vivo* setting. In our experiments, mast cell proteases induced detachment of endothelial cells from the basement membrane and from each other. It is possible that mast cell proteases induce detachment of living endothelial cells, as is the case with type IV bacterial collagenase, which is used for detaching endothelial cells for cell cultures[265]. Another potential explanation for the loss of endothelial cells is apoptosis. Indeed, recent data from our laboratory also show a role for mast cell proteases in mast cell-induced endothelial apoptosis (Heikkilä H. *et al.* unpublished results 2007). Moreover, *in vivo* mast cell-derived TNF-α may potentiate the proapoptotic effects of mast cell proteases[154].

Our experimental setting was far from ideal, albeit we consider it more physiological than *in vitro* models. The ideal way to show mast cell-induced endothelial damage in coronary arteries would be an experiment in which subendothelial mast cells would be stimulated and

the endothelium studied afterwards. However, this approach is difficult, as endothelial cells are damaged by mast cell-stimulating agents such as compound 48/80 directly[325]. It seems that the only way to stimulate subendothelial mast cells without damaging endothelial cells is via IgE-mediated hypersensitivity, which could be used only in animals but not in human studies. Unfortunately, we have not yet found a suitable animal model for studying this phenomenon, as at least mice, rats, and swine do not seem to have subendothelial mast cells in their coronary arteries. The notion that mast cell activation-related endothelial damage may indeed occur *in vivo* and may involve mast cell protease-mediated degradation of endothelial cell adhesion molecules is supported by the observed inhibition of radiologic contrast medium-induced endothelial damage by mast cell protease inhibitor[326].

Degradation of essential endothelial cell adhesion molecules by mast cell proteases

To study the mechanisms of mast cell induced endothelial erosion in more detail we conducted additional experiments. The results revealed that the mast cell proteases tryptase, chymase, and cathepsin G are all capable of degrading VE cadherin, which is a central molecule in the EC-EC adhesion. Mast cell proteases are all capable of degrading several basement membrane and extracellular matrix components as well as activating pro-MMPs, releasing matrix bound cytokines, and cleaving several plasma proteins (Table 5.). Furthermore, heparin protects cathepsin G[213], chymase[212], and tryptase[215] against inhibition. Thus, enzyme activity is prolonged in the presence of heparin and basement membrane proteoglycans, but remains strictly regulated at all times[327].

Table 5. The presently known and potential substrates of mast cell proteases which may be relevant in the pathogenesis of atherosclerosis.

Substrate	Tryptase	Chymase	Cathepsin G	Degradation product	Reference
Elastin	N.S.				
		N.S.			
			+	Fragments	328
Fibulin 1	N.S.				
		-			329
			N.S.		
Fibulin 2	N.S.				
		+			329
			N.S.		
Fibronectin	+/+			Fragments	330
		+/+		Fragments	331, 332
			+/+	Fragments	331, 333
Laminin	N.S.				
		N.S.			
			+/+		334-336
Nidogen	N.S.				
		+/ N.S.			337
			N.S.		
Tenascin	N.S.				
		N.S.			
			+/+		338, 339
Thrombospondin	N.S.				
		N.S.			
			+/+	Fragments	333
Type IV collagen	+/+				340
		+/+			332, 340
			+/+		340

Table 5. continues from previous page

Substrate	Tryptase	Chymase	Cathepsin G	Degradation product	Reference
Vitronectin	N.S.				
		+/+			341
			+/+	Fragments	331, 333
VWF	N.S.				
		N.S.			
			+/+		331, 333
VE-cadherin	+/ N.S.				Study III
		+/ N.S.			Study III
			+/+	Fragments	Study III and 103
Occludin	N.S.				
		N.S. /+		Fragments	342
			N.S.		
CD31	-/ N.S.				Study III
		-/ N.S.			Study III
			-/ N.S.		Study III
CD146	-/ N.S.				Study III
		-/ N.S.			Study III
			-/ N.S.		Study III
Complement 3	N.S.				
		N.S.			
			+	C3a + C3b	343
ICAM-1	N.S.				
		N.S.			
			+	Fragments	344
Pro-MMP-1	-				345
		+		MMP-1	345, 346
			+	MMP-1	347
Pro-MMP-3	+			MMP-3	345, 348
		+		MMP-3	345
			+	MMP-3	349
Angiotensinogen or Angiotensin I	N.S.				
		+		Angiotensin II	221
			+	Angiotensin II	82
Endothelin-1	N.S.				
		+		Fragments	350
			N.S.		
Factor VII	N.S.				
		N.S.			
			+		351
Factor IX	N.S.				
		N.S.			
			+		351
Factor X	N.S.				
		N.S.			
			+		352

Soluble substrate / *in vivo* or *in vitro* insoluble substrate. Symbols: + = cleaved by the enzyme, - = not cleaved by the enzyme, N.S. = cleavage by the enzyme not studied.

Potential role of mast cells in the induction of endothelial erosions causing acute thrombotic events

Taken together, our results and previously published studies suggest a role for mast cells in the induction of endothelial erosions. However, it is possible that mast cells induce endothelial cell apoptosis and/or detachment only under special conditions, i.e. when endothelial cells are weakened by additional factors, such as unfavorable flow conditions or toxic substances. The mechanisms by which mast cells may induce detachment or apoptosis of endothelial cells are summarized in figure 9.

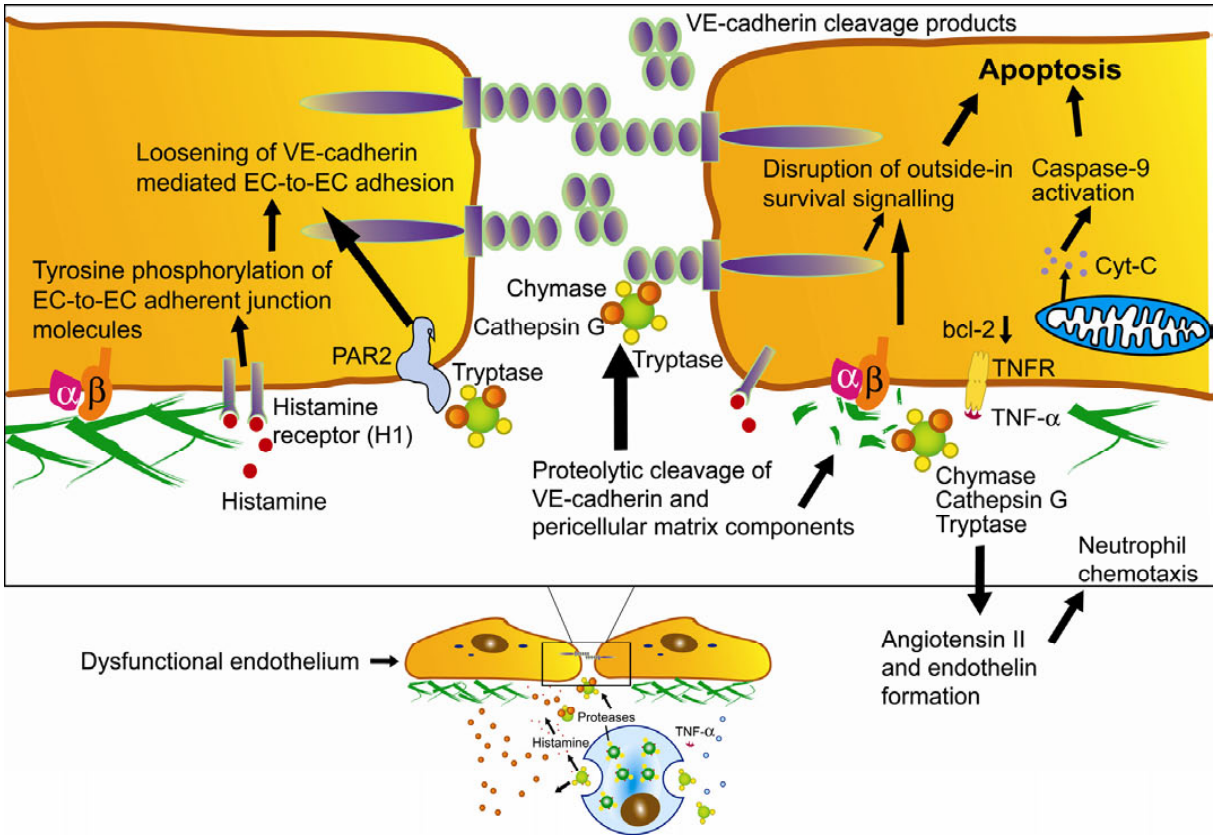

Figure 9. The proposed mechanisms of mast cell-mediated endothelial cell injury. Mast cells may weaken the adhesion of endothelial cells to the intima by releasing proteases capable of degrading basement membrane components. Furthermore, mast cell mediators induce loosening of endothelial cell-cell junctions. Taken together, these changes may lead to apoptosis or detachment of endothelial cells.

Importance of endothelial damage in atherosclerosis

Several independent reports have confirmed the presence of endothelial erosions in human arteries, especially in connection with human atherosclerotic plaques[273, 353]. These results are supported by reports showing circulating endothelial cells and endothelial cell-derived microparticles in patients with atherosclerosis[258, 354]. Endothelial erosions do not only predispose to acute thromboembolic events, but repeated healing of erosions by proliferation and migration gradually leads to endothelial cell senescence and loss of normal endothelial functions. Furthermore, erosions also lead to increased proliferation of underlying SMCs. Indeed, the duration of endothelial erosion has been reported to correlate directly with the extent of intimal SMC proliferation[250].

The importance of transient, minor endothelial damage in the pathogenesis of atherosclerosis is likely to be limited, as such damage is rapidly repaired[294]. However, if the damage is long-lasting or repeated, it is more likely to lead to atherosclerotic changes of the arterial wall[355]. It appears that several proatherogenic factors, including hyperlipidemia, hypertension, tobacco smoking, and recurrent infections, are all capable of causing minor endothelial damage. Albeit none of these factors cause acute denudation, they may induce endothelial dysfunction rendering endothelial cells more vulnerable to other noxious stimuli. Proatherogenic factors may also hinder endothelial regeneration by reducing the numbers of circulating endothelial progenitor cells[290].

The factors leading to impaired regeneration of endothelium are inadequately known. It is known that an increased rate of endothelial erosions and ensuing endothelial cell proliferation eventually lead to endothelial cell senescence, and senescent endothelial cells are commonly seen in areas prone to atherosclerosis[291]. It is also known that proatherogenic factors decrease telomerase activity and may thus promote endothelial cell senescence[287]. Senescent endothelial cells exhibit a reduced capacity to produce nitric oxide and a reduced capacity to proliferate[305].

4.4. Symptom status of carotid plaques is not determined by endothelial cell apoptosis (IV)

In previous studies endothelial cell apoptosis has been associated with endothelial erosion, thrombus formation and atherosclerotic plaque destabilization[356], and increased endothelial cell replication has been speculated to be the reason for endothelial cell senescence at the predilection sites of atherosclerosis[291]. Together, these previous observations motivated us to study the relationship between endothelial cell apoptosis and proliferation, endothelial erosions and thrombotic cerebrovascular events in human carotid plaques collected from asymptomatic and symptomatic patients. In a previous study using the same plaque material, large endothelial erosions on the plaques of symptomatic patients were observed in immunohistochemical analysis[357]. Furthermore, the results of a RNA microarray analysis of a subcohort of the same plaques suggested that apoptosis might be involved in the formation of these erosions. In this study, we verified the immunohistochemical observation of endothelial erosions on carotid plaques by scanning electron microscopy. We also noted numerous small thrombi at the sites of endothelial erosions (Figure 10). The balance between the apoptosis and proliferation of endothelial cells was investigated in plaques using immunohistochemical stainings and TUNEL assay.

Apoptosis and proliferation of endothelial cells

More endothelial cells positive for active caspase-3 were detected in plaques of asymptomatic patients (4.6% ± 0.7% of total EC count) than in plaques of symptomatic patients (3.3% ± 0.7%, p=0.049). However, proliferating ECs were also more common in asymptomatic patients, as shown by Ki-67 staining, and the number of Ki-67-positive cells correlated with the number of cells positive for active caspase-3 (r_s=0.275, p=0.040) suggesting active renewal of endothelium in asymptomatic patients. The notion of active caspase-3 positive staining represents apoptotic cells, was supported by TUNEL assay, although TUNEL stained ~40% more cells than active caspase-3. This is probably due to the fact that in addition to apoptotic cells, TUNEL may also stain necrotic cells and cells actively repairing DNA[358]. Interstingly, the number of apoptotic endothelial cells appeared to be higher in patients with high blood total cholesterol or LDL-C levels and lower in patients with high HDL-C levels (r_s=0.310, p=0.009). Diabetic patients also had more endothelial cells positive for active caspase-3 (r_s=0.230, p=0.049).

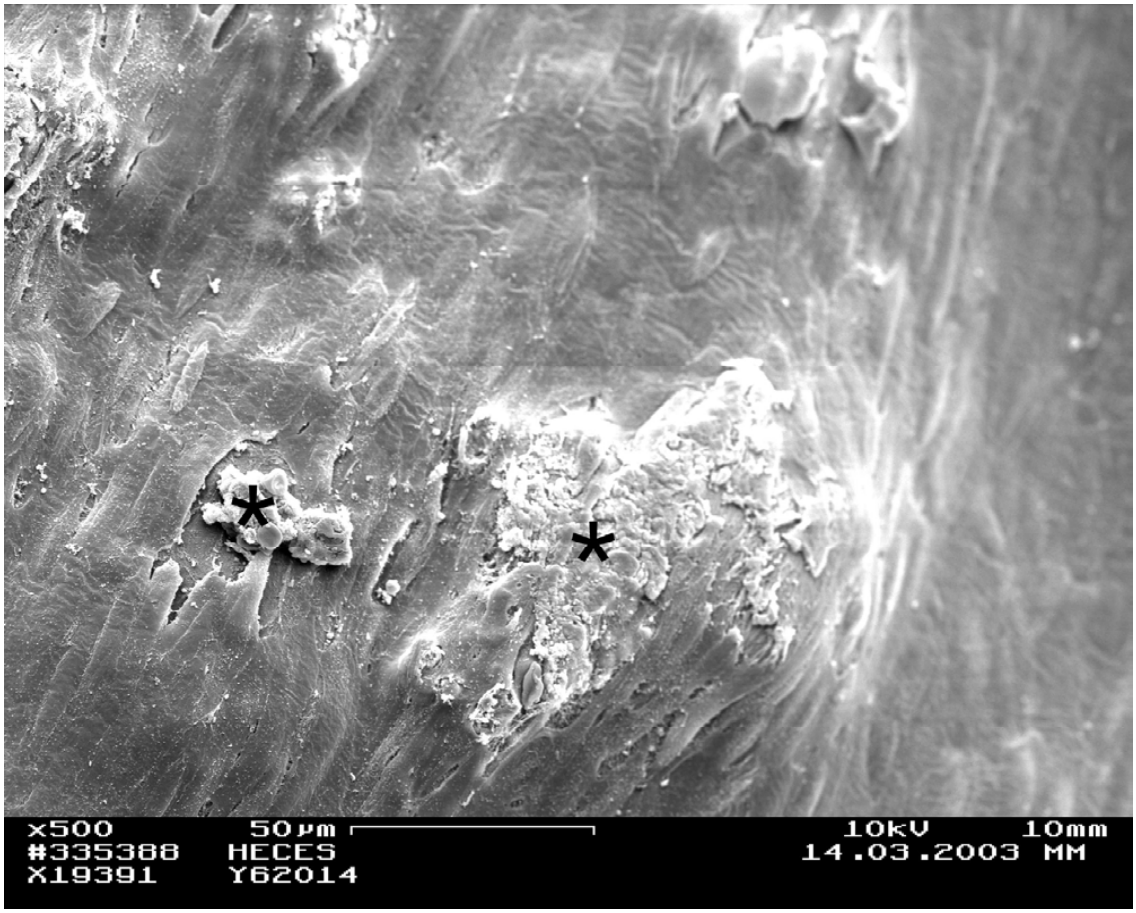

Figure 10. A representative scanning electron micrograph showing two small thrombi (asterisks) at sites of endothelial erosion on a human carotid artery plaque.

Low turnover and large erosions are associated with symptomatic plaques, whereas high turnover is associated with asymptomatic plaques

The results of this study confirm the previous reports of large denuded areas in complicated human carotid plaques[273, 359] and are in line with the current idea of the harmful impact of increased endothelial cell death and desquamation on plaque endothelium. However, the results also suggest that renewal of endothelium by cell proliferation may balance the harmful effects of endothelial cell apoptosis in asymptomatic patients, whereas in symptomatic patients the imbalance between apoptosis and proliferation leads to large endothelial denudations and ensuing thromboembolic complications. The present results do not fully explain the mechanisms leading to endothelial erosion, regardless of whether it consists of apoptosis, detachment of living endothelial cells, or decreased procreation of endothelial cells. However, the results underline the importance of the extent of endothelial erosions as the major determinant of symptom status.

Our results suggest that the risk factors of atherosclerosis, such as atherogenic plasma lipids (i.e. high LDL-C and low HDL-C) and diabetes, may increase the risk of imbalance between cell death and proliferation. This observation is also supported by numerous previous reports[65, 360].

4.5. Identification of endothelial erosions by staining simultaneously endothelial cells with CD31/34 and platelets with CD42b (V)

The rationale for this study was the observation of thin, endothelium-resembling platelet thrombi covering sites of endothelial erosion in the course of Study III. On the basis of an extensive literature search we were unable to find an antigen that would be specific for endothelial cells and suitable for reliable identification of small endothelial erosions of atherosclerotic plaques. The evaluated antigens were unsuitable, because they are expressed by cells or structures potentially covering sites of erosion or by cells directly underlying erosions, or they are not expressed by all endothelial cells. In this study we show that thin platelet layers may be hard to distinguish from endothelial cells in immunostainings when common endothelial cell markers are used. For most purposes this is not a problem, but when the goal is to detect minute endothelial erosions, it is a potential pitfall. Thus, a method for reliable simultaneous detection of endothelial cells and platelets is necessary.

On the basis of the literature search, we chose CD31/CD34 staining for indentifying endothelial cells as it identifies reliably all endothelial cells[361]. However, as shown by immunostainings of adjacent human coronary sections, CD31/CD34 staining also recognizes platelets which were identified with CD42b staining. With Western blotting, we were able to show that CD31 is expressed by both endothelial cells and platelets, whereas CD42b is not present in endothelial cells. The CD34 antibody used by us recognized only the CD34-antigen in endothelial cells, as the platelets and endothelial cells express different glycoforms of CD34[362]. Finally, we were able to show that it is possible to stain both endothelial cells and platelets simultaneously with fluorescent markers and to identify reliably even small endothelial erosions covered by microthrombi. Thus, the novel staining protocol presented in this study makes it easier to reliably detect platelet microthrombi and endothelial erosions in the future.

Interestingly, the stainings done with the new staining method clearly showed, that some platelet thrombi attached to the subendothelial matrix are positive for CD31, while some others are not. At this moment we can only speculate on the possible mechanisms causing the absence of CD31. Possible mechanisms including internalization, conformational change like dimerization/oligomerization[363], or antigen cleavage[364] should be investigated in future studies. Whether this change in the platelet CD31 reflects some difference in the functional status of platelets, e.g. different activation mechanism, different stage of thrombus maturation, or exposure of thrombus to high shear stress[364], remains also to be shown.

4.6. Pharmacological inhibition of mast cell-induced coronary constriction (unpublished results)

Coronary vasospasm has been speculated to increase risk of plaque erosion and rupture[260]. Interestingly, mast cell histamine[155, 365] and mast cell derived leukotrienes are capable of causing vasospasm of atherosclerotic arteries[366]. Furthermore, both antihistamines and leukotriene antagonists are widely used in the treatment of allergies, and leukotriene receptor antagonists have even been proposed as antiatherosclerotic drugs[367]. These previous findings motivated us to study the potential of histamine and leukotriene receptor antagonists in the prevention of mast cell induced coronary vasospasm. To our knowledge, this is the first study in which stimulation of living mast cells has been used for inducing coronary vasospasm. In our small series of organ bath experiments with human coronary artery rings, ~70% of the

mast cell-induced vasoconstriction could be abolished with the preincubation of coronary rings with histamine H_1-receptor antagonist diphenhydramine, whereas the cysteinyl leukotriene receptor antagonist ICI-198,615 had only a minor inhibitory effect (~2% of total mast cell-induced vasoconstriction) (Figure 11).

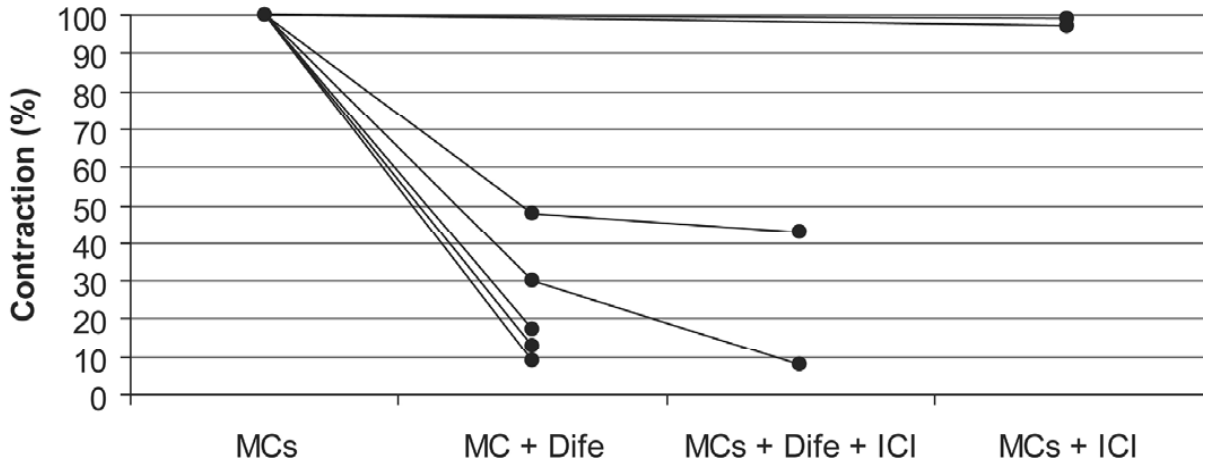

Figure 11. Inhibition of mast cell-induced contraction of atherosclerotic coronary arteries by the histamine H_1-receptor antagonist diphenhydramine (1 µM, Dife) and the leukotriene receptor antagonist ICI-198,615 (1 µM, ICI). Fresh human coronary rings obtained from recipient hearts at cardiac transplantations were equilibrated for 60 min at +37°C with 1.5 g resting tension prior to measurements. The force of contraction was measured with an isometric force-displacement transducer and registered on a polygraph (FT 03 transducer and Model 7E polygraph; Grass instruments, Quincy, MA, USA). Freshly isolated living rat peritoneal mast cells (~60 000 cells/ml) were pipeted into the organ bath vial and stimulated with the compound 48/80 (1 µg/ml). Unstimulated mast cells or compound 48/80 alone did not have any effect on vascular tone. Each line represents a coronary artery used for measurements. The contractions caused by mast cell degranulation were comparable to those obtained with 125 mM KCl. The function of endothelium was assessed by adding 1 µM acetylcholine in 1 µM noradrenalin-precontracted rings. The rings which dilated in response to acetylcholine (i.e. had functional endothelium) were considered non-atherosclerotic, and the ones which contracted (i.e. had dysfunctional endothelium) were considered atherosclerotic. Importantly, only the atherosclerotic arteries (i.e. the ones with dysfunctional endothelium) responded to mast cell-derived mediators with contraction.

5. SUMMARY AND CONCLUSIONS

Many lines of evidence support a role for mast cells in the pathogenesis of atherosclerosis. The purpose of the current study was to investigate the connection between mast cells and endothelial erosions in the context of atherosclerosis. On the basis of our results, the following conclusions can be presented:

1. Our results and other current evidence indicate that intraluminal administration of the vasodilator papaverine induces endothelial damage in arterial grafts. Papaverine-induced endothelial damage is likely to occur via two main mechanisms, i.e. by acid pH and by the formation of sharp needle-like papaverine precipitates. Also, papaverine-induced degranulation of mast cells may contribute to the formation of endothelial erosions. Our results corroborate the previous reports showing impaired endothelial function after use of papaverine on the morphological level[368]. However, as discussed in the chapter titled "Clinical relevance" on page 36, the clinical significance of the observed endothelial damage remains unclear, and clinical studies evaluating the short- and long-term effects of graft endothelial damage should therefore be initiated. Studies comparing the functional and morphological effects of different vasodilating substances on endothelial cells and SMCs should also be performed.

2. We show that the number of carotid plaque mast cells is high in patients with an atherogenic serum lipid profile. These results suggest that mast cells may be involved in the pathogenesis of carotid atherosclerosis. The association between mast cells and atherogenic serum lipids supports an earlier observation of increased mast cell counts in obese patients[122]. Furthermore, mast cells were commonly observed in areas occupied by T cells, and mast cell counts correlated with T cell density. These observations suggest a potential interaction between mast cells and T cells in carotid plaques, as has also been suggested for other diseases[131].

3. On the basis of our results, mast cells are associated with endothelial erosions in human coronary artery specimens. They may induce endothelial erosion *ex vivo*, and they are the main local source of cathepsin G in human coronary arteries. Our results also suggest that, in addition to the previously described mast cell-mediated mechanisms, which may loosen the EC attachment, degradation of VE-cadherin by mast cell serine proteases may also be involved in the detachment of ECs. These results provide novel insights into the mechanisms of mast cell-induced endothelial leakage in some clinical situations, including stroke or contrast medium-related blood-brain barrier leakage and radiologic contrast medium-induced microvascular leakage in lungs[108, 326]. Similar mast cell dependent mechanisms seem also to play a role in the regulation of gut epithelial permeability, which appers to be necessary for effective helminth expulsion[369, 370]. On the basis of this information, novel therapeutic approaches can be envisioned for these conditions.

4. Endothelial erosion is a major factor contributing to symptoms caused by carotid plaques. The present results suggest that there is a dynamic balance between denuding mechanisms, such as apoptosis and detachment of ECs, and regenerating mechanisms, such as adhesion of EC progenitor cells and proliferation of ECs on the luminal surface of human atherosclerotic plaques. Thus, future studies should determine the mechanisms leading to impaired endothelial regeneration, as inhibition of these mechanisms might be of importance in

preventing the progression and complications of atherosclerosis. Furthermore, therapeutic strategies enhancing endothelial regeneration should also be developed.

5. On the basis of our results, a combination of scanning electron microscopy and conventional light microscopy seems to be a reliable method for evaluating endothelial damage. However, one has to be careful when interpreting the results of EC immunostainings the purpose of which is to show endothelial erosions. This is because ECs and platelets share a large number of common antigens and may thus resemble each other in immunostainings. Our results show that adhered platelets may be mistaken as endothelial cells, and many endothelial erosions may thus remain undetected if a platelet-specific antibody is not used together with the EC markers. This pitfall may be overcome by using the double immunostaining protocol described here. Our results do not explain the molecular mechanisms responsible for the presence of CD31-positive and negative thrombi. Thus, the physiological relevance and the mechanisms behind this observation remain to be elucidated in future studies.

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Heikki Mäkynen is thanked for introducing me to the mysteries of testing endothelial function in an organ bath system. Albeit not very fruitful, this collaboration was scientifically a very educating experience for me.

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Helsinki, April 2007

Mikko Mäyränpää

7. SELECTION OF REFERENCES

This literature review has been written solely for the purpose of introducing the reader to the research relevant to this work. It is not meant to cover all aspects of relevant research in detail, but rather to give an overview of it.

Many of the references in this literature review are review articles, as it was necessary to limit the number of references, and as some of the conclusions presented can only be drawn from numerous publications combined together. When discussing the specific areas of interest of the substudies of this thesis, the articles describing previous original findings are cited.

The search for references has been done by:

1. Systematic literature searches from PubMed and various library databases available via the RefWorks database.
2. Searching for relevant articles with the “related articles” option of PubMed and RefWorks.
3. Searching relevant articles in the reference lists of the articles and books read by the author.
4. Searching the internet and internet databases.

When searching, the known synonyms of the relevant keywords were used. Boolean operators and other limiting options of the search engines were used to limit the search results to the most relevant articles.

The following principles were used to accept a reference:

1. The reference had to be an article in a peer-reviewed journal or published by some other source considered reliable (such as Statistics Finland).
2. The reference (or at least its abstract) had to be available in English. References with the main text written in a language other than English were used only when no other suitable references were available.
3. When two or more otherwise equally suitable articles were available on the same topic, the references readily available via the internet or local library collections were preferred over ones that were more difficult to obtain.
4. Unpublished data on recent findings from our laboratory have been cited in a few places.

8. ONLINE DATABASES

These online databases have been used as a source of information:

Databases provided by the National Center for Biotechnology Information.
http://www.ncbi.nlm.nih.gov/

iHOP - Information Hyperlinked over Proteins.
http://www.pdg.cnb.uam.es/UniPub/iHOP/

The ExPASy (**Ex**pert **P**rotein **A**nalysis **Sy**stem) proteomics server of the Swiss Institute of Bioinformatics (SIB).
http://au.expasy.org/

The Human Protein Reference Database.
http://www.hprd.org/

The genecards database.
http://www.genecards.org/

MEROPS - the Peptidase Database.
http://merops.sanger.ac.uk/

Brenda, The Comprehensive Enzyme Information System.
www.brenda.uni-koeln.de

The Gene Ontology Database.
http://www.geneontology.org/

The library collections available via RefWorks.
http://www.refworks.com/

KEGG: Kyoto Encyclopedia of Genes and Genomes.
http://www.genome.jp/kegg/

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